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Response in Breast Cancer Metastasis

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13. ABSTRACT (Maximum 200) Breast cancer cell growth response to transferrin (Tf) and expression of the transferrin receptor (TfR) were found to correlate with metastatic capability. To test this observation, Tf responsiveness or TfR expression were increased in selected poorly-metastatic breast cancer lines, through means of selection and transfection processes. This resulted in increased metastatic behavior in certain low metastatic rat breast cancer cell types. Studies into the differences in Tf-induced intracellular responses by the altered and parental lines have been initiated. For further experiments of this nature, additional plasmid constructs containing the human TfR cDNA in sense and anti-sense orientation were developed. To investigate the consequence of lab-created autocrine secretion of Tf by breast cancer cells, a new eukaryotic expression vector containing the Tf cDNA was made. Novel assays to aid in metastasis detection and cellular Tf binding were developed. These results and techniques will aid in the future study of breast cancer cell heightened response to Tf.				
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FOREWORD

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Philip Cavanaugh 11/20/97
PI - Signature Date

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Cavanaugh, P.G., and Nicolson, G.L. The selection of a metastatic rat mammary adenocarcinoma cell line from a low metastatic parental population by an in vitro process based on cellular ability to proliferate in response to transferrin. *Journal of Cellular Physiology* (In Press).

pages 18 - 31.

Cavanaugh, P.G., Jia, L., and Nicolson, G.L. Rat MTLn2 mammary adenocarcinoma cells transfected with the transferrin receptor gene exhibit an enhanced response to transferrin and an increased metastatic capability. *Breast Cancer Research and Treatment* (submitted).

pages 32 - 47.

INTRODUCTION

We had previously performed a number of studies to explore the mechanism whereby certain rat 13762NF breast cancer sublines preferentially metastasized to the lung. One of the hypothesis contemplated was that the metastatic cells had become competent to respond to lung-situated growth factors. In support of this, we found that media conditioned by viable, perfused rat lung fragments possessed mitogenic activity for lung-metastasizing cells, but no such activity in the media was evident for non-lung metastasizing cell lines from the same tumor system (1,2). The responsible lung-metastatic breast cancer cell proliferation-inducing factor was purified from the conditioned media (1,2) and was found to be transferrin (Tf; 3).

Transferrin is an iron-binding and transport protein which mediates the delivery of iron into cells via binding to the cell surface transferrin receptor (TfR; 4,5). The proliferative effect of transferrin on cells is thought to be due primarily to its iron transport function, which results in the maintenance of key enzymes required for proliferation, such as ribonucleotide reductase (RR; 6).

Our previously mentioned results led us to investigate the TfR expression on a number of breast and other cancer cell lines. Here, we found a correlation between the expression of this receptor and published metastatic capability (7-10). In exploring the growth response of various tumor cell lines to transferrin, we noted that certain high-TfR expressing lines exhibited no Tf-induced proliferative activity (7,11). Therefore, high TfR expression alone did not endow cells with a transferrin-mediated proliferative response.

Others have found that the expression of TfR in human tumor cells correlates with tumor grade, stage, progression, and metastasis in breast carcinomas (12,13), bladder transitional cell carcinomas (14), and malignant melanoma (15). In addition, high levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (16), and the expression of TfR was higher in a human melanoma line selected for metastatic capability in nude mice than in the poorly metastatic tumor cells of the parental population (17). Other investigators have also observed that tumor cell proliferative response to transferrin correlates with metastatic capability and that transferrin-responsiveness may be involved in secondary site-specific tumor cell growth (18-20).

We have also shown that the metastatic capability of certain poorly-metastatic breast cancer cell lines can be increased by up-regulating tumor cell transferrin receptor expression through means of selection or transfection processes (9,10). In these studies, the rat MTLn2 mammary adenocarcinoma line was exposed to a selection process whereby only those cells that could survive in serum-free media containing low levels of transferrin were culled from the parental population (9). When compared to the parental cell line, the selected cells were found to possess increased numbers of cell surface TfR, to demonstrate a markedly enhanced proliferative response to transferrin, and to metastasize to a greater extent to the lymph nodes and lungs of syngeneic F344 rats (9). When MTLn2 parental cells were transfected with the cDNA encoding the human TfR (hTfR) and sorted for high hTfR expressors, the sorted, transfected line expressed 10 - 15 fold more total TfR than the parental line and possessed a uniquely sensitive capability to proliferate in response to transferrin in serum-free conditions (10). This line also demonstrated an ability to metastasize to the lungs of nude mice to a greater degree than did the vector-transfected parental (10).

The purpose of the grant was to expand on the observations mentioned in the first paragraph. The accomplishments so far are mentioned in the fifth paragraph. Additional efforts are being performed to improve upon these and to explore some of the unusual findings revealed by the studies completed to date.

BODY:

A.) Selection of highly metastatic rat MTLn2 mammary adenocarcinoma cells from the poorly-metastatic parental population by an in vitro process based on cellular ability to survive and proliferate in low serum containing media supplemented with low levels of transferrin.

Work in this area has been reported on extensively in the past. New data generated this year on this system concerning signal transduction will be discussed later in the report. A manuscript concerning the results with this experiment was submitted for publication this year. New data concerning the basic selection and metastasis story was generated only in response to reviewer's requests. The paper was accepted and is now in press in the *Journal of Cellular Physiology*. Since the manuscript is slightly altered from previous versions and presents the results in a more organized form, it has been included in the appendices as a reprint.

Methods: MTLn2 cells were placed in a stress culture situation where all cells normally died. If this stress culture was supplemented with low levels of rat transferrin, a few colonies were seen to arise, presumably due to their ability to use transferrin in such a way as to ensure their survival and proliferation. These colonies were harvested and exposed to twenty like cycles of culture in these conditions.

Results: When compared to the parental population, the final selected cells displayed increased numbers of transferrin receptors (TfR), an increase ability to grow in response to transferrin (Tf), and an increased ability to form lung metastases in rats.

Discussion: The results indicate that those few cells in a mixed parental breast cancer population which possess the ability to utilize transferrin as a sole survival and proliferation agent, are the more aggressive, metastatic cells. In some systems, ability to respond to transferrin is an earmark of highly metastatic breast cancer cells.

B.) Transfection of poorly metastatic rat MTLn2 mammary adenocarcinoma cells with the gene encoding the transferrin receptor results in an increased ability of those cells to proliferate in response to transferrin and an increased ability to metastasize to the lungs of nude mice.

The majority of these results were also presented in previous progress reports. In those reports, the line was stated to be human MDA231 cells. However, I became suspicious about the cells originally supplied to me. Concerns about the total lack of human TfR expression on these and differences between them and MDA231 cells acquired from the ATCC led me to further investigate their nature, where I found them to be rat in origin. Further characterization uncovered their identity as being from the MTLn2 line. However, the system was still usable and the results obtained support the basic hypothesis. Further experimentation was performed and the manuscript concerning this work was re-drafted. Since significant changes were made since last year's version, a copy of this is also included in the appendix.

Methods: A eukaryotic human TfR expression plasmid was made by excising the TfR cDNA from pcDTR1 and cloning it into pcDNA1Neo. Rat MTLn2 cells were transfected with this and high human TfR expressors were selected by FACS. The sorted cells were analyzed for growth response to Tf, for Tf internalization capability, for cell surface TfR expression, and cell surface Tf binding. The cells were tested for their ability to metastasize to the lungs of nude mice from a primary mammary fat pad tumor site.

Results: all properties measured were increased as a result of the transfection process. It was found that the transfected population formed twice as many lung metastases as did the vector transfected controls, and that the lung tumor burden in the former was 6-7 fold higher than in the latter.

Discussion: These results also support a role for transferrin responsiveness and TfR expression in the ability of certain breast cancer cells to metastasize.

C.) Formation of new plasmids:

The current pcDNA1/Neo TfR expression construct, is a large plasmid which does not elicit high TfR expression in all cell types. A full length anti-sense TfR construct, also cloned into pcDNA1/Neo, has not resulted in the down-regulation of TfR in cells transfected with it. I have long desired to create new constructs using the shorter pcDNA3.1 plasmid as a vector, and to create shorter length anti-sense TfR constructs. Also, it was desired to produce a transferrin expression vector, with the hopes that autocrine secretion of transferrin would be realized in cells transfected with this.

Methods: new TfR plasmid: The TfR coding region was removed from pcDTR1 (21) using EcoRV and Xba I. The fragment was isolated by agarose electrophoresis and electroelution, and cloned into the MCS of pcDNA3.1 (InVitrogen, San Diego, CA).

TfR antisense plasmid: The TfR coding region was further digested with Kpn I; the resulting fragment possessed an Eco RV site at the 5' end and a Kpn I site at the 3' end. The MCS of pcDNA3.1 has novel Kpn I and Eco RV sites oriented in the 5' - 3' direction, respectively. The abbreviated TfR fragment was ligated into pcDNA3.1 cut with the same two enzymes.

Tf expression plasmid: The Tf coding region was cut from R27A (22, 23) using Fsp I and Ssp I, producing a blunt end DNA fragment. This was cloned into Eco RV cut and alkaline phosphatase treated pcDNA3.1. Minipreps were made from ampicillin-resistant, ligation product-transformed E. coli colonies. Plasmid DNA was assessed for sense or antisense Tf cDNA inserts by cutting minipreps with Xba I and Pst I. Both constructs were found. Colonies were expanded and plasmids isolated for transfection studies.

Discussion: all new constructs formed as expected. Analytical re-digestion of aliquots of the various constructs produced DNA fragments of the expected size (Figures 1 and 2). As pcDNA3.1 is a preferred eukaryotic expression vector, transfection studies with these will hopefully result in successful increased expression of TfR or Tf using sense vectors, and decreased expression of TfR using the antisense vector.

D.) Preliminary experiments examining Tf-dependent production of phospho-tyrosine residues in Tf-responsive and Tf-non-responsive breast cancer cell lines.

Introduction: Some initial investigations into possible signal transduction events elicited by Tf in our Tf-responsive mammary adenocarcinoma lines have been performed. I have conducted preliminary investigations into the appearance of Tf-induced phospho-tyrosine residues in Tf stimulated, Tf-responsive (and non-responsive) breast cancer lines, and have found some indication of low-M_r Tf-enhanced Tyr-P bands in MTLn2/TfR cells (figure 3).

Methods: Cells were be serum starved for 2d. Cells were then placed in growth assay plates, in serum-free conditions. When 0.2 - 0.5 µg/ml of rat or human holo-Tf is added to these, MTLn2/TfR and MTLn2-Tf20 display a growth response whereas MDA-468, MTLn2, and MTLn2/Neo do not. At various time points pre- and post- Tf addition, cells were lysed in RIPA (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, and 100 U/ml aprotinin), loaded onto SDS-PAGE gels at equal protein amounts, electrophoresed, blotted, and blots were assessed for tyrosine-phosphate (Tyr-P), moieties by application of anti Tyr-P antibody followed by an appropriate HRP-conjugated second antibody and ECL. Use of the MTLn2/Neo and MTLn2/TfR lines were emphasized since these are from the same parental

source, vary tremendously in their response to Tf (pre-print 2), and possess the same morphology and growth rates in stock culture. Preliminary results of some of these experiments are shown in figure 3.

Discussion: The difference in Tf responsiveness between the transfected MTLn2, MDA-MB-468 and A375p cells despite high TfR expression by all indicates that a transferrin-dependent cell division process is initiated in certain tumor cells which is dependent on mechanisms other than the quantity of TfR present and the amount of Tf-TfR complexes extant on the cell surface. This series of experiments is designed to attempt to delineate these processes, since it is those which seem to deliver metastatic competence to breast cancer cells.

E.) Analysis of ribonucleotide reductase (RR) levels in Tf-responsive and Tf-nonresponsive breast cancer lines.

Methods: An antibody which recognizes RR on Western blots has been located. The assay is a straightforward blot one sharing many procedures with methods listed in part C. Equal cell equivalents of starved Tf stimulated or non-stimulated cell lysates are electrophoresed, blotted, incubated with anti-RR, and HRP-conjugated anti-mouse IgG. Sites of HRP localization are detected by ECL.

Discussion: This study is the beginning of those designed to determine if Tf-responding cells are able to do so because of continued high expression of the iron-requiring proliferation dependent RR enzyme. Preliminary results have indicated a possible up-regulation of the enzyme in a responding cell line when compared to a non-responding line (Figure 4).

NEW TECHNIQUES:

F.) Analysis of Tf binding using FITC-Tf, Western blotting, and fluorescent image analysis.

Methods: Human holo-FITC-Tf was obtained from Molecular Probes. Cells were plated in twelve well plates and grown to 60 - 70 % confluence. Cells were rendered Tf-deficient by two changes in α -MEM only for 2h each. Blocking solution (DMEM containing 2 mg/ml liquid gelatin) was added to all wells. Cells were then equilibrated to 4 °C. Increasing levels (0.025 - 0.1 μ g/ml) of FITC-Tf were added to wells in triplicate and one well of each set also received a 200 fold excess of un-conjugated Tf. Cells were incubated at 4°C for 2 h and washed extensively with PBS. Cells were lysed in RIPA buffer and equal cell equivalents from each well were treated and loaded onto an SDS-PAGE gel. The samples were electrophoresed and transferred to a PVDF membrane. The membrane was blocked and incubated with alkaline phosphatase conjugated anti-FITC. The membrane was washed extensively, was incubated with a fluorescent alkaline phosphatase substrate, dried, and quantitated on a STORM imager.

Results: Cell bound Tf was easy to quantitate using this technique and an obvious fluorescent signal was produced at the correct molecular weight for Tf. Cell bound Tf was increased in the TfR transfected line when compared to the vector transfected control. All bound signal was eliminated when excess un-conjugated Tf was included, indicating that the binding was specific (Figure 5).

Discussion: This procedure is one where radiolabeled ligand is replaced by FITC-conjugated ligand, for traditional tracking of ligand binding and internalization using Scatchard analysis and other procedures. The detection system is fairly sensitive, using a Western blot technique to measure the amount of ligand bound and to verify its molecular weight. The MTLn2/TfR cells produced excellent results here, however, they express high numbers of transferrin receptors.

The universality and sensitivity of the system has not yet been tested using a different ligand known to bind to cells at low levels.

G.) Specific immunohistochemical detection of rat breast cancer cells in nude mouse lungs.

Rat MTLn2 breast cancer cells formed few micrometastases in nude mice. Detection of these in hematoxylin and eosin staining of lung sections was a touch and go process. To facilitate visualization of the lesions, a technique which would cause them to stand out in ordinarily processed formalin fixed paraffin embedded tissues was desired. A number of antibodies were screened for their ability to do this, and one advertised to recognize rat and human cytokeratin 17 seemed to function.

Methods: Lungs were removed from nude mice, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Blocks were sectioned at 5 μ m thickness and resulting slides stored at 25°C. Sections were de-paraffinized in two changes of xylene, equilibrated in two changes of absolute ethanol, treated with 10% H₂O₂ (in methanol) for 30 min., placed in 10 mm citric acid (pH 6.0), and exposed to 5 min heating in a microwave oven. Sections were washed in PBS and incubated in a block solution (PBS containing 5% v/v goat serum and 2 mg/ml gelatin) for 30 min. All subsequent antibody incubations were performed in block solution and washes in PBS occurred between all steps. Slides were incubated overnight at 4°C in 1:100 normal mouse IgG₁ or anti Cytokeratin-17 (Chemicon, Temecula, CA), for 2 h at 25°C in 1:100 biotin-conjugated goat anti-mouse IgG (Sigma), and for 1 h at 25°C in 1:100 streptavidin-HRP (Sigma; 25°C, 1h). Slides were incubated in DAB substrate (Sigma) for 5 min., washed, equilibrated in absolute ethanol followed by xylene, coverslipped, and examined by phase contrast microscopy.

Discussion: Rat tumor cells stained dark brown with this antibody, whereas normal mouse lung tissue was essentially unstained with the exception of some light brown coloration in the bronchial epithelia. Small micrometastases (10 - 20 cells in diameter) stained uniformly brown (Figure 6) whereas larger tumors (200 - 300 cells diameter) displayed more of a heterogeneous pattern.

CONCLUSION:

The major hypotheses of the grant have been proven true, but only for the rat MTLn2 cell line. Other lines have exhibited increased TfR levels via transfection with that same TfR construct, however, a corresponding increase in growth response to Tf was not observed. In those cases, *in vivo* metastasis assays have not yet been performed, as I have desired to see that proliferation response prior to expending the time and expense on animal studies. It is hoped that with the new TfR encoding plasmid, that higher TfR levels will be achieved and at least one other breast cancer cell line (preferably human) will respond as the MTLn2 cells have. The discovery of cellular factor(s) which differentiate between cells which demonstrate a proliferative burst in response to Tf versus those that don't seems to be the most important point to be addressed. The Tf-encoding plasmid has finally been produced, after a number of unsuccessful attempts. Four lines are currently transfected with this. It is hoped that data concerning the task to be performed with this construct will be acquired soon.

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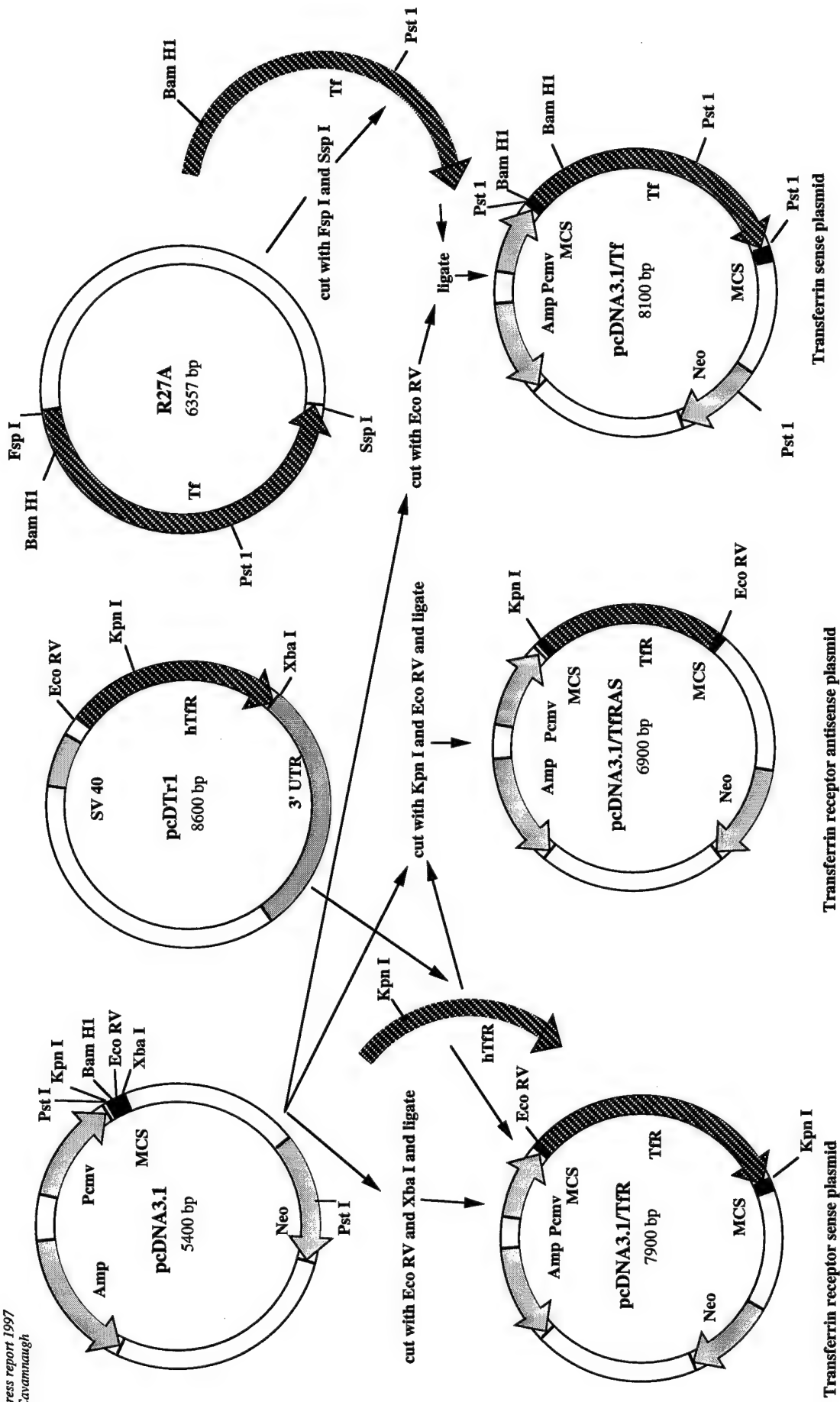


Figure 1A: Schematics of the formation of three new plasmids. pcDNA3.1 was the "recipient" vector for all. It was cut with Eco RV and Xba I to receive the sense TfR fragment, with Xba I and Kpn I to receive the TfR antisense fragment., and with Eco RV only to receive the Tf coding region.

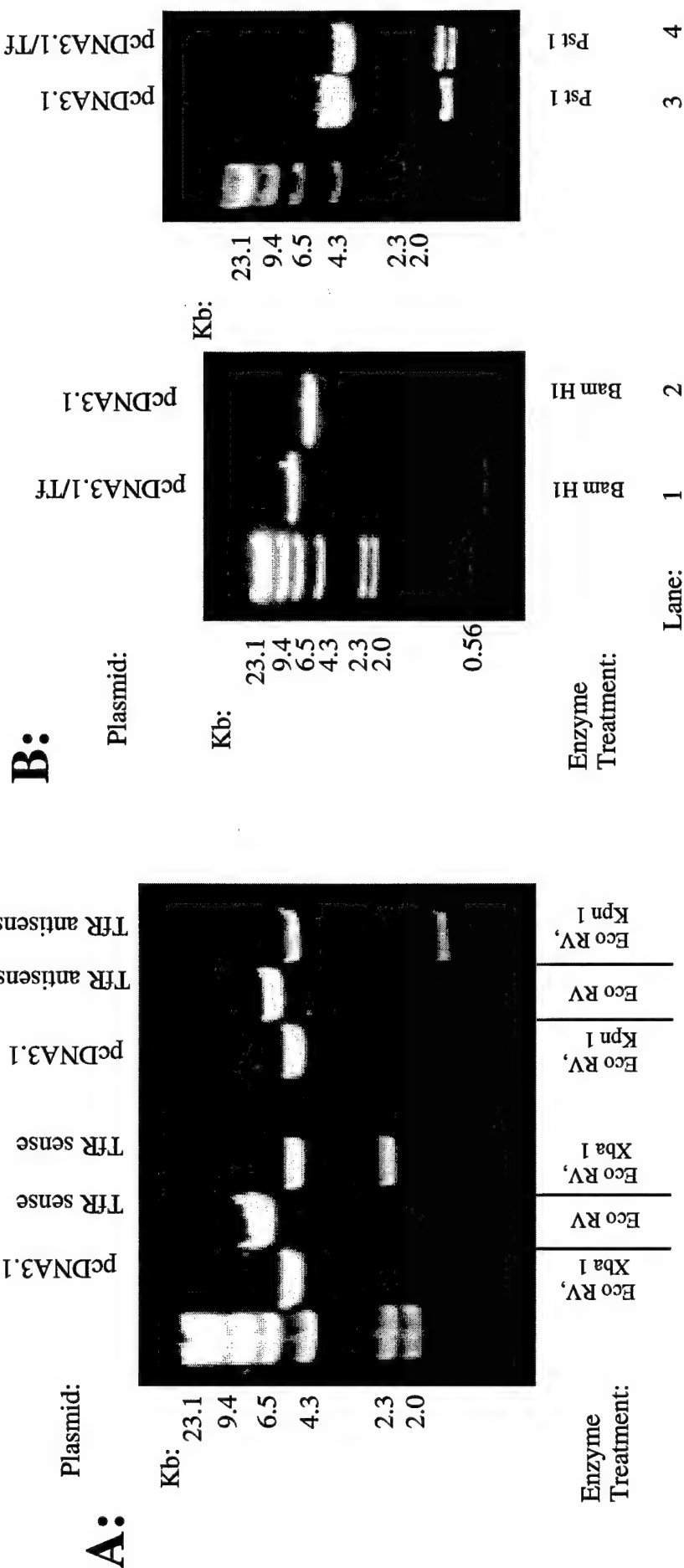


Figure 2. Results of endonuclease treatment of new plasmids.
A: *Eco RV* and *Xba I* cuts pcDNA3.1 displayed one band at 5.4 Kb (lane 1), and pcDNA3.1/TfR displayed the 5.4 Kb vector and the 2.5 Kb insert (lane 3). When linearized with *Eco RV* only, the TfR plasmid displayed one 7.9 Kb band (lane 2).
Eco RV and *Kpn I* cuts: pcDNA3.1 again displays one band at 5.4 Kb (lane 4); the TfR antisense plasmid produced two bands: the 5.4 Kb vector and the 1.5 Kb insert (lane 6). When linearized with *Eco RV* only, the latter plasmid displayed one band at 6.9 Kb (lane 5).
B: when linearized with Bam HI, pcDNA3.1 produced one 5.4 Kb band (lane 2); the /Tf plasmid generated a large 7.8 Kb and a small 0.3 Kb fragment (lane 1). Upon Pst I cleavage, pcDNA 3.1 produced two bands, at 4.0 and 1.4 Kb (lane 3), and the /Transferrin construct displayed those bands plus 1.5 and 0.8 Kb fragments resulting from the ligation (lane 4).

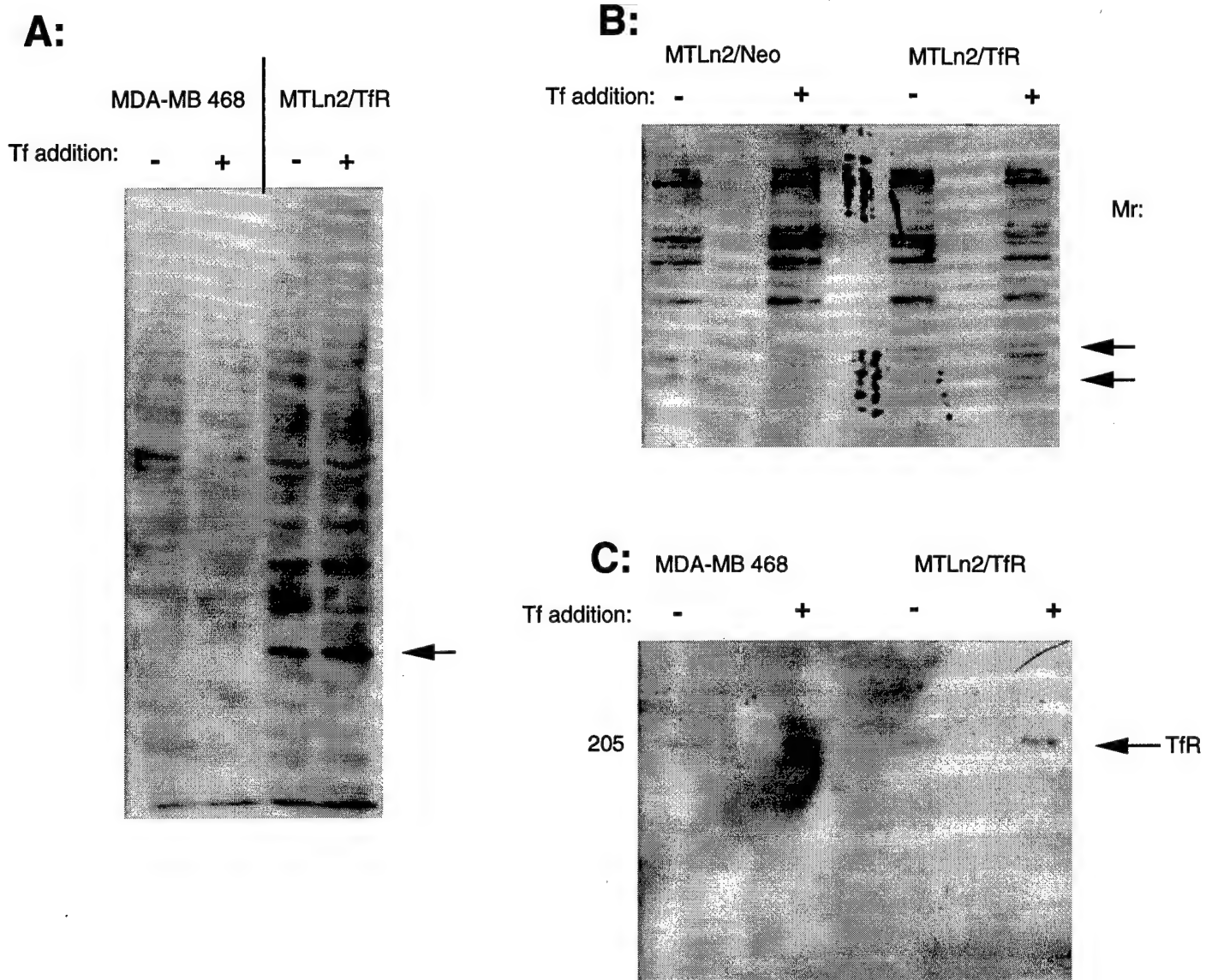


Figure 3: Results of analysis of the Tf-responsive MTLn2/TfR cells and Tf-nonresponsive MTLn2/Neo and MDA-MB 468 cells for the Tf-induced presence of phospho-tyrosine containing proteins. All cells were serum-starved for 2d, by multiple changes of serum-free media. With the final change, some wells of each cell type received media containing 1 μ g/ml human Tf. 12h later, cells were washed, lysed, separated by SDS-PAGE and analyzed for Tyr-P by western blotting. Equal protein amounts were loaded onto each lane. **A:** A possible Tf-induced up-regulation of one Tyr-P containing protein was seen (arrow) in MTLn2/TfR cells, which was absent in the non-responsive MDA-MB 468. **B:** A group of low Mr Tfr-P containing bands were seen in the MTLn2/TfR cells which were fainter in the MTLn2/Neo cells (arrows). However, Tf-induced up-regulation was questionable. **C:** Analysis of Tyr-P in Tfr immunoprecipitates of MDA-MB 468 and MTLn2/TfR cells. The only band seen was Tfr itself and both lines appeared to exhibit a Tf-induced up-regulation.

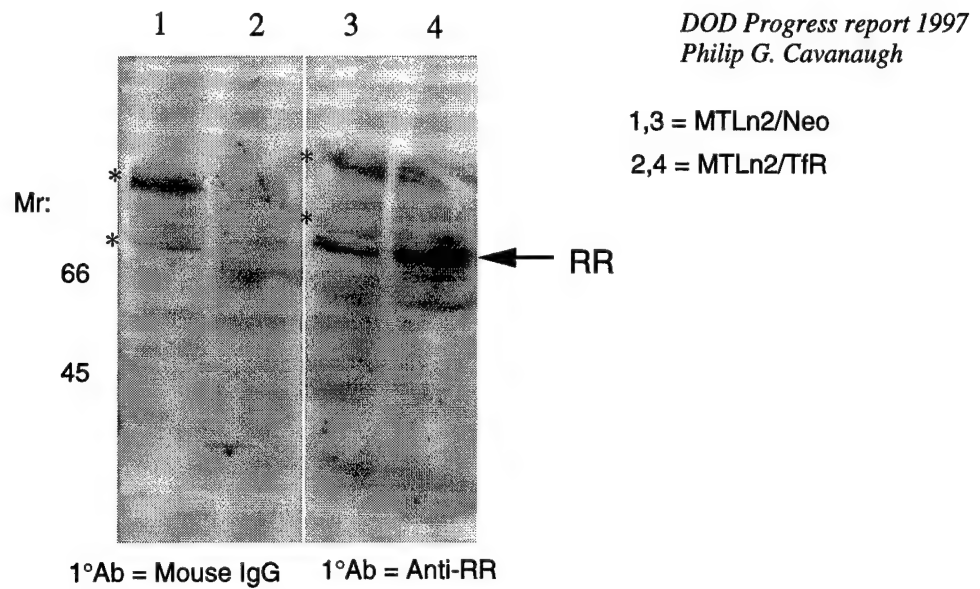


Figure 4: Possible evidence of increased levels of ribonucleotide reductase (RR) in MTLn2 cells transfected with the hTfR gene and thereby rendered highly responsive to Tf. Stock cultures of MTLn2/Neo or MTLn2/TfR cells growing logarithmically in media plus 5% FBS were washed, lysed, separated by SDS-PAGE and analyzed for RR by Western blotting and ECL. Equal protein amounts of lysates were loaded onto all lanes. The antibody used (clone AD203, Biogenesis, Sandown, NH) recognizes the 70 Kd RR M1 subunit. Bands labeled with an * are non-specific IgG binding bands.

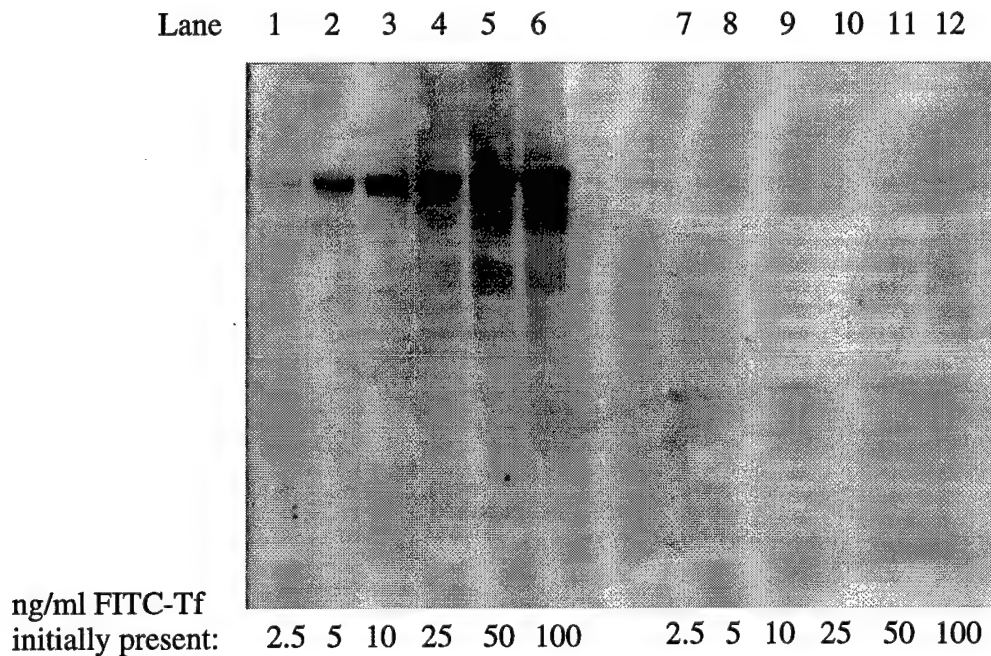


Figure 5. Measurement of FITC-Tf binding in MTLn2/TfR cells. Cells at 50 - 60% confluency growing in 12 well plates were serum-starved, then treated at 4° C with increasing levels of FITC-Tf. After 2h, cells were washed, lysed, and equal cell equivalents were electrophoresed, blotted, incubated with anti-FITC, anti-mouse IgG-AP, and the Atto-Phos AP substrate. The blot was then analyzed using a STORM imager. Lanes 1-6 = lysates from cells exposed to the concentration of FITC-Tf listed above. Lanes 7 - 12 = lysates from cells treated as in 1-6, but with a 200-fold excess of un-conjugated Tf.

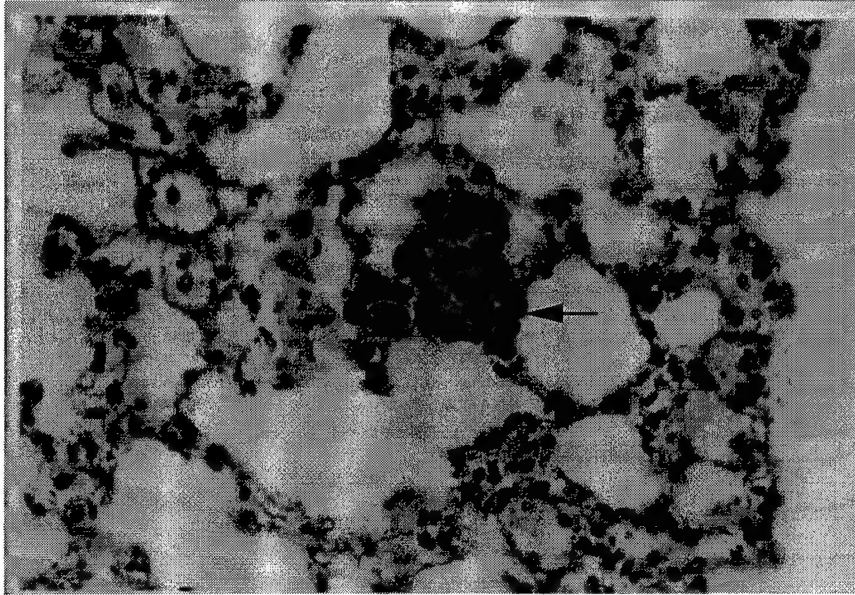


Figure 6. Representative micrograph of an MTLn2/TfR micrometastasis in a nude mouse lung section. The formalin fixed, paraffin embedded tissue was sliced at a thickness of 5 μ M, de-paraffinized, unmasked using a citrate buffer, and incubated with mouse anti-cytokeratin17. This was followed by an incubation with biotinylated anti-mouse IgG and an incubation with streptavidin-HRP. An insoluble DAB HRP substrate was applied, the section was dehydrated and examined by phase contrast microscopy. The metastasis is indicated by the arrow.

ANIMAL USE REPORTING

The University of Texas M.D. Anderson Cancer Center

Activity Name & Address: 1515 Holcombe, Houston, TX 77030

Contract number: DAMD17-94-J-4189

Philip G. Cavanaugh, P.I.

Animal Type Genus/Species	Animals Purchased or Bred	Animals Used	USDA Pain Column C	USDA Pain Column D	USDA Pain Column E	AAALAC Accreditation
Nu/Nu mice	30	30		X		A3343-01

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**Selection of highly metastatic rat MTLn2 mammary adenocarcinoma cell variants
using *in vitro* growth response to transferrin**

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Running Head: Tumor cell transferrin response and metastasis.

7 text figures and 1 table.

Abstract

We previously found that the proliferative response to transferrin and the expression of transferrin receptors (TfR) on the cell surface of various rat 13762NF mammary adenocarcinoma cell sublines correlated with their spontaneous metastatic capability. To further assess the involvement of transferrin and TfR in metastasis, transferrin-responsive cells were selected from a poorly-metastatic 13762NF subline (MTLn2) with poor response to transferrin. When maintained in low serum (0.3%) conditions, MTLn2 cells failed to survive. However, if the medium was supplemented with 0.5 µg/ml rat transferrin, some colonies survived, probably because of their ability to proliferate in response to the added transferrin. The surviving cells were expanded and exposed to 10 or 20 similar cycles of transferrin growth selection to obtain the sublines MTLn2-Tf10 and MTLn2-Tf20, respectively. The MTLn2-Tf20 cells proliferated in response to transferrin at a rate similar to that of the high metastatic 13762NF sublines. Using immunofluorescent staining, Scatchard analysis, and affinity isolation of TfR, we discovered that the MTLn2-Tf20 cells had 5 to 6 times more TfR than did the parental MTLn2 line. When injected into the mammary fat pads of rats, the MTLn2-Tf20 line metastasized to the axillary lymph node in 7 out of 10 animals and to the lungs in 6 out of 10 (median number = 13). No metastases were seen in the MTLn2 parental line. The MTLn2-Tf10 cells showed intermediate properties compared with the MTLn2 and MTLn2-Tf20 cells. The results indicate that variant cells with a high response to transferrin may be more metastatic than the bulk cells in a poorly metastatic population. The selection of cells with high levels of TfR and a higher proliferative response to transferrin results in sublines with greater potentials for spontaneous metastasis.

Introduction

An important property of metastatic cells is thought to be their ability to respond to paracrine growth factors found in target organs for metastasis formation (Nicolson, 1988a, 1988b). In previous studies we explored the possibility that the metastasis of tumor cells to a target organ was enhanced, in part, by the ability of the malignant cells to respond to growth factors encountered in the micro-environment of that organ. For example, we examined cell lines derived from the rat 13762NF mammary adenocarcinoma for their proliferation in response to medium conditioned by lung fragments (Nicolson, 1988c, Cavanaugh and Nicolson, 1989). A growth response was seen only in those sublines that had a high propensity for lung metastasis formation. A tumor cell mitogen purified from such medium and partially sequenced was found to be the iron transport protein transferrin (Cavanaugh and Nicolson, 1990; 1991). Subsequent studies showed a correlation between tumor-cell response to transferrin and metastatic capability in 5 out of 6 tumor model systems in animals and humans (Nicolson, 1988c; Cavanaugh and Nicolson, 1990, 1991, Inoue *et al.*, 1993).

Transferrin binds to a specific cell surface receptor, the transferrin receptor (TfR)³, a $M_r \sim 180,000$ homodimeric integral membrane glycoprotein (Testa *et al.*, 1993), that can bind two iron-saturated transferrin molecules and is responsible for the delivery of iron into cells either through internalization of iron-transferrin (Laskey *et al.*, 1988) or activation of a NADH-dependent oxidoreductase associated with the plasma membrane that mediates the trans-plasma membrane transport of iron from transferrin (Thorstensen and Romslo, 1988). Rapidly dividing cells, including various of tumor cells (Testa *et al.*, 1993; Neckers and Trepel, 1986; Shindelman *et al.*, 1981), usually express high levels of TfR. The expression of TfR in human tumor cells, as determined by histochemical analysis, correlates with tumor grade, stage, progression, and metastasis in breast carcinomas (Wrba *et al.*, 1986), bladder transitional cell carcinomas (Seymour *et al.*, 1987, 14), and malignant melanoma (Van Muijen *et al.*, 1990). In addition, high levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (Yoda *et al.*, 1994). The expression of TfR was higher in a human melanoma line selected for metastatic capability in nude mice than in the poorly metastatic tumor cells of the parental population (Van Muijen *et al.*, 1991). Transferrin receptor expression on nonsmall cell lung cancers has been reported to be an indicator of poor prognosis in certain groups of patients (Whitney *et al.*, 1995), and transferrin was found to be the major mitogen in bone marrow for prostatic carcinoma cells with a high affinity for metastasis in bone marrow in humans (Rossi and Zetter, 1992). In animal tumors, the proliferative response to transferrin is associated with malignant progression in a series of murine B16 melanoma sublines (Nicolson *et al.*, 1990, Stackpole *et al.*, 1995).

The proliferative effect of transferrin on cells is thought to be due primarily to its ability to transport iron into the cell, thus maintaining the activity of key enzymes required for proliferation, such as ribonucleotide reductase (Testa *et al.*, 1993). However, some studies have suggested that iron transport alone cannot

explain the growth stimulating activity of transferrin (Kovar and Franek, 1989; Sanders, 1986; Sirbasku, *et al.*, 1991). Also, iron delivered by transferrin mediates processes that soluble iron cannot (Alacantha *et al.*, 1991 25), and stimulation of TfR through transferrin-independent means induces several activities in T cells (Salmeron *et al.*, 1995).

We modulated tumor-cell response to transferrin using a selection technique that allowed the survival of only those rat mammary adenocarcinoma cells that responded to low levels of transferrin. We found that the metastatic capability was markedly greater in the transferrin-selected cell populations than that in the parental line.

Materials and Methods

Cells and Cell Culture. The rat 13762NF mammary adenocarcinoma was originally developed in Fischer 344 rats by dietary administration of 7,12-dimethylbenz[a]-anthracene; the MTLn2 line was originally cloned from a lung metastasis arising from a 13762NF tumor growing in the mammary fat pad (Neri *et al.*, 1982). MTLn2 cells displayed little or no metastatic ability upon injection into syngeneic F344 rats (Neri *et al.*, 1982; Welch *et al.*, 1983). The MTLn2 line was maintained at 37°C in a humidified 95% air - 5% CO₂ atmosphere in α -MEM containing 5% (vol/vol) fetal bovine serum (FBS). The line was routinely tested for mycoplasma contamination and found to be uncontaminated.

Rat holo-transferrin preparation. Rat transferrin (apo-form; Sigma, St. Louis, MO) was dissolved at 1 mg/ml in 25 mM sodium bicarbonate, pH 8.0, containing 1 mg/ml ferric ammonium citrate. After incubation for 1 h at 25°C, the solution was dialyzed extensively (2 X 4 liters) against 25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5.

Selection Procedure. Cells were removed from stock plates with 0.25% trypsin, 2 mM EDTA (in Ca²⁺, Mg²⁺ free phosphate-buffered saline [PBS]), and seeded onto 100-mm cell culture plates at a density of 20,000 cells/plate in 10 ml of α -MEM containing 1% FBS. One day later, the medium was changed to 10 ml of α -MEM containing 0.3% FBS, and rat holo-transferrin was added to selection plates at a final concentration of 0.5 μ g/ml. The control plates received only the corresponding amount of Tf solvent (25 mM HEPES-buffered Dulbecco's modified eagle's medium [DMEM], pH 7.5, containing 10 mg/ml bovine serum albumin [BSA]). Seven days later, the cells were removed from the plates with 0.25% trypsin, 2 mM EDTA, and re-seeded onto 100-mm cell-culture plates at a density of 2,000 cells/plate. The selection process was then repeated 10 times. For selection rounds 11-20, FBS was eliminated from the medium.

Cell Growth Assays. The cells were removed from stock plates and seeded in 96-well plates in 100 μ l of α -MEM containing 1% FBS at a density of 2,000 cells/well. One day later, the medium was changed to 100 μ l of α -MEM containing 0.3% FBS, and increasing amounts of rat holo-transferrin was added to the test wells. Five days later, the cells were quantitated using a crystal violet stain assay as follows: The cells were washed with PBS, and then fixed at 25°C with PBS containing 5% (vol/vol) glutaraldehyde. After 30 min, the cells were washed with water, allowed to dry, and then stained for 30 min at 25°C with 50 μ l 0.1% crystal violet in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 9.5. The stained cells were washed with water and then solubilized with 50 μ l of 10% acetic acid. Cell numbers were determined by measuring absorbance at 590 nm on a Dynatech model MR5000 plate reader (Chantilly, VA). Absorbance in this system directly correlates with cell numbers up to \approx 50,000 cells/well (Cavanaugh and Nicolson, 1990; Keung *et al.*, 1989).

Immunofluorescent Detection of Cell Surface TfR. Cells grown on LabTek slides (Nunc, Naperville, IL) were washed three times with PBS and equilibrated to 4°C. The primary antibody (anti-rat TfR, clone OX-26, BPS, Indianapolis, IN) or normal mouse IgG were each diluted 1:100 at 4°C in PBS containing 10 mg/ml of BSA (PBS-BSA). The antibody or IgG solution was added to the cells, and the slides were incubated on ice for 2 h. The cells were washed three times with PBS-BSA and then incubated on ice with a 1:100 dilution (in PBS-BSA) of an Fab' fragment of phycoerythrin-conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA). The cells were washed three times with PBS-BSA and examined for fluorescence using a Nikon Diaphot phase contrast microscope (Melville, NY).

Fluorescent-activated cell sorting (FACS) analyses. All cell washes and antibody incubations were performed using 25 mM HEPES-buffered DMEM (pH 7.5) containing 10 mg/ml BSA (DMEM-BSA). The

cells were removed from culture plates using 0.25% trypsin, 2 mM EDTA (in Ca^{2+} , Mg^{2+} -free PBS). As soon as detachment was complete, FBS was added to 1% (vol/vol), to neutralize the trypsin. The cells were washed twice by centrifugation, resuspended in DMEM-BSA and counted. The cell density was adjusted to $1 \times 10^6/\text{ml}$, and the cell suspensions were equilibrated to 4°C. Normal mouse IgG or anti-rat-TfR (final dilution = 1:100) was added to the cell suspensions, and the cells were then incubated at 4°C for 1 h, washed twice with DMEM-BSA, and incubated at 4°C with a 1:100 dilution of an Fab' fragment of phycoerythrin-conjugated rabbit anti-mouse IgG. The cells were washed twice with DMEM-BSA and then analyzed for fluorescence using a Becton-Dickinson FACScan instrument (San Jose, CA).

Spontaneous Metastasis Assays. All experimentation involving animals was approved by an Institutional Animal Care and Use Committee, and animals were treated and housed under conditions specified by the NIH, the Department of Health and Human Services and the Department of Agriculture. Cultured cells were removed from culture plates with calcium, magnesium-free hanks basic salt solution (CMFH) containing 1 mM EDTA and 0.25% trypsin. The cells were washed three times with CMFH, and cell density was measured using a model ZM Coulter Counter (Coulter, Hialeah, FL) and adjusted to $5 \times 10^6/\text{ml}$ with CMFH. The cells were kept at room temperature and were injected in a 0.2-ml volume into the left mammary fat pad (2 cm anterior to the hind leg) of Metofane (Methoxyflurane; Pittman-Moore, Washington Crossing, NJ) -anesthetized syngeneic female F344 rats (age range: 6 - 8 weeks). Six weeks later, the animals were killed with an overdose of Metofane and examined for gross metastatic lesions. In addition, lungs of certain animals were fixed in buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined for micro-metastases.

Affinity Isolation of TfR using Immobilized Tf. Cyanogen bromide-activated agarose was washed with 10 volumes of 1 mM HCl and equilibrated in a coupling buffer consisting of 0.1 M NaHCO_3 , 0.5 M NaCl, pH 8.3. Apo-transferrin was added to the agarose in coupling buffer at a ratio of 10 mg of protein per ml of packed gel. The mixture was incubated at 4°C overnight in an end-over-end mixer. The gel was treated with 1 M ethanolamine, pH 8.0, for 2 h at 25°C and was washed with coupling buffer, then with 0.1 M acetate, pH 4.0, containing 0.5 M NaCl, and again with coupling buffer. The immobilized Tf was iron saturated by exposure to 1 mg/ml ferric ammonium citrate in 0.1 M NH_4HCO_3 . The gel was washed with PBS containing 0.5% (vol/vol) Triton X-100 (Van Driel *et al.*, 1984; Turkewitz *et al.*, 1988). To reduce inherent bound Tf, cells were incubated in two changes of α -MEM only (2 h each) before the analysis. The cells (70-80% confluent in 100-mm dishes) were then washed three times with 5 ml of PBS (4°C), and 3 ml of PBS containing 1.0 mg of Biotinamidocaproate N-hydroxysuccinimide ester (Sigma, initially dissolved in 0.2 ml DMSO) was added. The dishes were incubated at 4°C for 90 min while being shaken. The cells were then washed five times with 5 ml of PBS and lysed in 3 ml/dish of PBS containing 2.0% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N- α -p-tosyl-L-lysine chloromethyl ketone, 0.1 mM iodoacetamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin, at 4°C for 2 h. The lysate was centrifuged at 13,000 X g for 10 min. Lysate supernatant protein was determined using the detergent compatible BCA assay (Pierce, Rockford, IL). The lysate supernatant (1-2 mg of total protein) was combined with an excess of transferrin-agarose (0.4 ml of packed gel) and incubated for 2 h at 37°C. The gel was harvested by centrifugation at 2,000 x g and washed three times by suspension in PBS containing 2% Triton X-100. The centrifugation step was then repeated. Bound-cell-lysate proteins were released by exposure of the gel to 0.4 ml of nonreducing sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment solution at 95°C for 10 min. The samples were separated on a 7.5% SDS-PAGE gel according to Laemmli (1970) and blotted onto Immobilon membranes (Millipore, Bedford, MA) using a 10 mM CAPS, pH 10.0, transfer buffer. The membrane was blocked for 2 h at 25°C with PBS containing 10% nonfat dry milk and 0.5% Tween 20 and then incubated with blocking solution containing a 1: 2,000 dilution of streptavidin-horseradish peroxidase (streptavidin-HRP; Boehringer Mannheim, Indianapolis, IN) for 1 h at 25°C. The membrane was washed four times with 40 ml of PBS containing 0.5% Tween 20, and HRP-enhanced chemiluminescence (ECL) substrate (DuPont, Wilmington, DE) was applied. Light-emitting bands were detected by autoradiography and quantitated using a Hoeffer (model GS300 San Francisco, CA) scanning densitometer.

Scatchard analysis of cell surface TfR. To 1 ml of a 1 mg/ml solution of Rat holo-transferrin (in 25 mM HEPES, pH 7.5) was added 0.5 MCI of Na^{125}I (ICN, Cleveland, OH) and 10 μl of a 10 mg/ml solution of chloramine-T. This mixture was incubated at 25°C for 1 h, and 10 μl of a 20 mg/ml sodium bisulfite solution was added. The sample was passed through a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated and run with PBS. One milliliter fractions were collected, and aliquots of each fraction were

examined for radioactivity using a gamma counter. Radioactive fractions corresponding to the void volume were pooled. Specific activity was $\approx 300,000$ cpm/ μ g protein. Tumor cells were grown in 12-well plates to 70-80% confluence. To reduce inherent bound transferrin, the cells were incubated in two changes of α -MEM only (2 h each) before the analysis. Cells were washed twice with PBS, and 1 ml PBS containing 1% (vol/vol) liquid gelatin (Sigma) was added as a non-specific blocking agent. The plates were equilibrated to 4°C and increasing levels of 125 I-rat holo-transferrin (from 0.33 to 6.0 μ g/well) were added (in multiples of five) to wells. Immediately prior to the addition of transferrin, two wells at each dose received a 200-times excess of unlabeled transferrin. The wells were incubated for 2 h at 4°C and then washed five times with PBS. The cells were lysed with PBS containing 2% Triton X-100, lysates were placed into scintillation vials, and cell-bound counts were determined using a Packard A5550 gamma counter (Packard, Meriden, CT).

Results

When poorly metastatic rat MTLn2 mammary adenocarcinoma cells were cultured at 20,000 cells per 10-cm dish in α -MEM containing 0.3% FBS, none of the cells survived. However, if a similar culture was supplemented with 0.5 μ g/ml of rat transferrin, 2 to 10 colonies of surviving cells appeared. When these cells were harvested and re-exposed to the same transferrin selection conditions, 10 to 30 colonies appeared. As the selection cycles continued, increasing numbers of surviving cells were found in the cultures. After 18 to 20 cycles of selection, the selected cell subpopulation produced a nearly confluent culture. Furthermore, during the last 10 cycles of selection for transferrin-responsive cells, FBS could be eliminated from the system. With each cycle of selection, however, all cells failed to survive in transferrin-free medium. After 20 cycles of selection, an MTLn2 cell population was obtained (MTLn2-Tf20) that demonstrated an enhanced ability to proliferate in response to transferrin when compared with the parental MTLn2 cells (Fig. 1). Both lines grew at the same rate in the standard culture conditions using α -MEM supplemented with 5% FBS (Fig. 2).

We next determined if the amount of cell-surface TfR was increased in the MTLn2-Tf20 cells. Immunofluorescent microscopy analysis of cell surface TfR using anti-rat TfR (clone OX-26) followed by phycoerythrin-conjugated anti-mouse IgG revealed visibly greater cell surface fluorescence in the selected MTLn2-Tf20 subpopulation than in the parental MTLn2 cells (Fig. 3). We also analyzed the expression of cell surface TfR by FACS. The cells were rapidly removed from plates with trypsin/EDTA, stained for TfR at 4°C, and analyzed for fluorescence. This analysis indicated that there was 6.8 times more TfR on the MTLn2-Tf20 line than on the MTLn2 line (Fig. 4). In addition, this procedure revealed that the MTLn2-Tf10 line expressed a level of TfR between that of the MTLn2 and MTLn2-Tf20 lines, indicating that TfR per cell increased gradually during the selection protocol.

An additional procedure based on affinity isolation of TfR after biotinylation (29, 30) was used to quantitate cell-surface TfR in the cell lines. This procedure was performed after we found that the anti-rat TfR antibody used for immunofluorescent studies was unable to immunoprecipitate biotinylated TfR (data not shown). The cell surfaces were biotinylated, cells lysed with PBS containing Triton X-100, and the resulting solubilized cell material was exposed to immobilized transferrin. The agarose-transferrin preferentially bound to biotin-TfR in the lysate, which was released with SDS-PAGE sample buffer. The released TfR could be measured after SDS-PAGE separation followed by electrotransfer and detection of biotinylated bands by incubation of the blot with streptavidin-HRP followed by ECL. This method indicated that MTLn2-Tf20 cells had 5- to 6-fold more TfR per cell than did the parental MTLn2 cells (Fig. 5).

To verify the results of biotin-TfR affinity isolation and FACS and to assess any changes in the affinity for Tf in the two lines, conventional Scatchard analysis using 125 I-Tf was performed. Liquid gelatin was found to be an ideal blocking agent for this, and non-specific binding was reduced to near-background levels. In agreement with other assays, Scatchard analysis revealed that the MTLn2-Tf20 cells possessed 5 to 6 fold more surface TfR than did the MTLn2 cells. No significant differences in affinity for Tf were seen (Fig. 6).

To ascertain the metastatic behavior of both cell lines, spontaneous metastasis assays were performed. When 1×10^6 cells of either cell line were injected into the left mammary fat pad of syngeneic Fisher 344 rats, primary tumors formed at equal (70-80%) frequencies, and the tumors grew to a similar size. After six weeks of tumor growth, 7 out of 10 rats receiving the MTLn2-Tf20 cells had palpable left axillary lymph

node metastases (Table 1). Necropsy revealed that 6 out of 10 rats receiving MTLn2-Tf20 had lung metastases, but metastases were not evident in any of the animals injected with the parental MTLn2 line (Table 1). Representative photographs of lungs from animals injected with MTLn2 or MTLn2-Tf20 cells are shown in Fig. 7.

Another experiment was performed to compare the spontaneous metastatic capability of MTLn2 and MTLn2-Tf10 cells. The procedure was identical to that for the previous study, with the exception that six rats were used in each group. In this experiment, the MTLn2-Tf10 cells formed lung metastases in 1 out of 6 rats and left axillary lymph node metastases in 3 out of 6 rats. The only metastasis seen with the MTLn2 cells was an axillary lymph node metastasis in one animal (Table 1). Thus it appeared that the metastatic capability of the MTLn2-Tf10 line was between that of the MTLn2 and MTLn2-Tf20 lines. As the expression of cell surface TfR in the MTLn2-Tf10 line was also shown to be intermediate to that of the MTLn2 and MTLn2-Tf20 lines (Fig. 4), these results provided further proof that TfR expression correlated with metastatic capability in this system. Examination of lung sections for micro-metastases revealed that animals injected with MTLn2 cells possessed occasional diffuse sub-pleural micro-metastases. However, lungs from rats receiving MTLn2-Tf20 cells displayed extensive nodular metastases with large areas of lung replaced by tumor cells.

Cultures were established from the lung metastases from one animal injected with the MTLn2-Tf20 cells. When all cultured cells were re-analyzed for rat TfR expression by FACs, the results indicated that a maintenance of hierarchy of TfR expression was maintained, with mean fluorescence for MTLn2 being 65.7 that for MTLn2-Tf20 being 176.4 and MTLn2-Tf20 (metastases, second passage) equaling 199.8. No loss and perhaps even a slight gain of TfR expression was seen as the MTLn2-Tf20 cells metastasized. As lungs from rats receiving MTLn2 cells displayed no gross metastases, these samples were not cultured.

Discussion

Our previous results (Cavanaugh and Nicolson, 1989, 1990, 1991; Inoue *et al.*, 1993; Nicolson *et al.*, 1990 4-6,7,20) and those from other laboratories (Seymour *et al.*, 1987; Van Muijen *et al.*, 1990, 1991; Yoda *et al.*, 1994; Rossi and Zetter, 1992; Stackpole *et al.*, 1995) have indicated that in many malignant cell systems, the proliferative response of tumor cells to transferrin and expression of TfR correlates with metastatic capability. The observation of a cellular property associated with and perhaps thought to be responsible in part for metastatic properties has frequently resulted the use of *in vitro* selection techniques to isolate tumor cells with high or low levels of a metastasis-associated marker or activity. The influence on metastatic behavior can then be determined by assessing the metastatic capability of the selected cells *in vivo*. These types of experiments have resulted in the identification of a number of tumor cell properties thought to be associated with the ability to form metastases (Tulberg and Burger, 1985; Kalebic *et al.*, 1988; Ishikawa *et al.*, 1988; Brodt, 1989; Katz and Witz, 1993; Tressler and Nicolson, 1989; LaBiche *et al.*, 1993).

The differential expression of TfR and response to transferrin seen in metastatic cells naturally raised the question as to whether the metastatic behavior of tumor cells could be altered by artificially manipulating the transferrin/TfR system. A number of processes were considered where transferrin responsiveness and TfR expression could be enhanced in a poorly metastatic cell line to create a high metastatic line or reduced in a highly metastatic cell line to decrease its metastatic capability. We had previously performed Tf growth-response assays (Cavanaugh and Nicolson, 1989, 1990), where highly metastatic rat mammary adenocarcinoma cells proliferated in response to transferrin under low-serum conditions, whereas poorly metastatic cells proliferated to a much lesser degree or not at all. A logical method to select transferrin-responsive cells from a low metastatic population, then, was to scale-up the transferrin growth assays into an *in vitro* selection process whereby those few cells from the poorly metastatic population that responded to Tf could be isolated, expanded, and re-selected.

The poorly metastatic MTLn2 line (originally isolated from a lung metastasis; Neri *et al.*, 1982) was the ideal choice for these studies because its growth rate under the usual culture conditions (minimum essential media, alpha modification [α MEM] containing 5% FBS) was equal to the more highly metastatic MTLn3 line (Neri *et al.*, 1982). Also, the MTLn2 line is totally unable to survive in low-serum or serum-free conditions. Other poorly metastatic lines from the 13762NF series (such as MTPa) were less well suited to the selection process because their basal growth rate was much lower than that of the high metastatic lines (Neri *et al.*, 1982), and they demonstrated greater survival properties in low serum conditions. This last

characteristic contributed background during the selection process. Finally, the rat 13762NF mammary adenocarcinoma was an ideal choice for this experiment because it is a syngeneic tumor system, and the various sublines generated from it were originally selected based on their different spontaneous metastatic capabilities (Neri *et al.*, 1982). Also, the metastatic spread of cells in the 13762NF series mimics the pathogenesis of mammary adenocarcinoma cells in humans, with an initial metastatic migration to the regional lymph node(s) followed by metastasis to other organs. Selection and test results with the MTLn2 line were obtained rapidly, because these cells replicate and form large primary tumors in a short time. Other poorly metastatic animal tumor cell lines from other tumor systems are currently being selected for high transferrin-responding cells in this same manner; however, those selections are currently in their early stages.

We have found that a series of metastatic rat MTLn2 mammary adenocarcinoma cells that have high numbers of TfR and the ability to proliferate in response to low concentrations of transferrin can be selected from a low TfR-expressing, poorly transferrin-responsive parental cell population. The selected cells demonstrated a much greater ability to form spontaneous axillary lymph-node and lung metastases in syngeneic rats than did the parental line. Our results confirmed previous observations and indicate that a tumor cell's ability to respond to transferrin can be an important characteristic for metastasis formation, at least in some tumor cell systems.

The existence of micro-metastases in the lungs of animals injected with MTLn2 cells indicates that some of these cells possessed the ability to initiate the metastatic process. The further progression of MTLn2-Tf20 cells to gross metastatic lesions in the animals receiving these may indicate that the enhanced ability to respond to transferrin may not be responsible for the initial formation of metastases, but for the rapid growth of these into larger lesions. Nevertheless, the results indicate a more aggressive metastatic behavior for the MTLn2-Tf20 cells.

Our results raise the question as to whether or not the amount of transferrin present in a target organ influences metastatic cell growth. Significant levels of transferrin can be thought to be circulating through any tissue, since a relatively high level of transferrin is present in blood. Whether or not other localized pools of transferrin exist in a given tissue that can exert an effect other than, or in addition to serum transferrin is questionable. The non-lactating mammary gland synthesizes some, but low levels of transferrin (Lee *et al.*, 1987; Grigor *et al.*, 1990) and its status as a site of transferrin localization is uncertain. As tumor growth in the mammary fat pad for both lines was equal, this might indicate that low levels of transferrin was localized there, or that tumor growth in the fat pad was a result of interaction with many other factors. Stimulated lymph nodes can be thought of containing high levels of transferrin, since macrophages situated in these synthesize transferrin needed for optimum lymphocyte growth (Djehe, *et al.*, 1993). Assuming tumor cell containing lymph nodes are stimulated, then this mechanism may play a role in high-TfR expressing tumor cell growth in the lymph node. This may explain why we found gross lymph node metastases in many rats injected with the MTLn2-Tf20 line, but not in those injected with the MTLn2 line. Previously, we found no evidence that perfused adult rat lung synthesizes transferrin (Cavanaugh and Nicolson, 1991). However, more transferrin seems to be found in the adult perfused lung than in many other organs. Rossi and Zetter (1992) report that extensively washed human adult lung contains more immuno-recognizable transferrin than does kidney, skin, bone marrow, or liver; and Meek and Adamson (1985) find more transferrin localized to adult perfused mouse lung than in any other tissue examined. This may explain why tumor cells with high TfR expression and ability to proliferate in response to transferrin form larger, more visibly apparent lung metastases.

Heightened proliferative response to transferrin does not necessarily explain the ability of these cells to preferentially metastasize to, or target the lung. Other unknown tumor cell factors such as those which might mediate increased adhesiveness to lung tissue or motility in response to lung components may also be involved. However, the possibility that the lung acts as a transferrin storehouse indicates that a transferrin/TfR-mediated mechanism for lung-targeting by tumor cells might exist.

We have not ruled out the possibility that increased expression of TfR and response to Tf may have other roles in metastasis formation. Other functions of transferrin have been described, such as its ability to promote angiogenesis by stimulating endothelial cell invasion and migration (Carlevaro *et al.*, 1997), and to effect the adhesion and migration of chicken mesoderm explants (Sanders, 1986).

As these more malignant cells are often the ones which develop resistance to therapy and lead to treatment failures and metastasis, a therapeutic regimen which initially targets such cells might be of value. Along these lines, reports on the ability of toxin conjugated transferrin, anti-transferrin receptor antibodies, iron chelators, or gallium salts which compete for transferrin binding sites against various tumors have been made. Success in this area has been limited to cancers of hematopoietic origin but suggest further research into such therapy for other tumor types.

The results further suggest that continued exploration of therapeutic strategies that interfere with transferrin binding or iron uptake by tumor cells (Elliot *et al.*, 1988; Kemp *et al.*, 1995) are warranted.

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Figure Legends:

Fig. 1. Proliferative response of MTLn2 and MTLn2-Tf20 cells to transferrin. Cells were plated in α -MEM containing 1% FBS at a density of 2,000 cells/well in 96-well plates. One day later, the medium was changed to medium containing 0.3% FBS and increasing amounts of rat holo-transferrin were added. Five days later, cell numbers were determined using a crystal violet stain procedure. Points represent the mean \pm SD of quadruplicate samples.

Fig. 2. Growth rate of MTLn2 and MTLn2-Tf20 cells under usual culture conditions (α -MEM containing 5% FBS). Cells were plated in α -MEM containing 5% FBS at a density of 2,000 cells/well in 96 well plates. Two and four days later, cell numbers were determined using a crystal violet stain procedure. Points represent the mean \pm SD of quadruplicate samples.

Fig. 3. Immunofluorescent analysis of cell surface TfR on (A) MTLn2 and (B) MTLn2-Tf20 cells. The cells were grown on LAB-TEK chamber slides and stained at 4°C with the OX-26 anti-rat TfR antibody followed by a phycoerythrin conjugated Fab' fragment of anti-mouse IgG. The cells were then examined for fluorescence using a Nikon Diaphot microscope. Phase-contrast microscopy indicated that the cells were of approximately equal density. Cells initially treated with an irrelevant mouse IgG produced no fluorescence (data not shown).

Fig. 4. Analysis of cell surface TfR using FACS. The cells were removed from the culture plates with trypsin/EDTA, stained at 4°C with the OX-26 anti-rat TfR antibody followed by a phycoerythrin-conjugated Fab' fragment of anti-mouse IgG, and were analyzed for fluorescence using a Becton-Dickinson FACstar instrument. An isotype-control fluorescence of 4 - 8 was obtained when any of these cell lines were initially incubated with normal mouse IgG (data not shown).

Fig. 5. Results of cell surface TfR measurement in MTLn2 and MTLn2-Tf20 cells by affinity isolation of biotinylated TfR. The cell surfaces were biotinylated at 4°C, the cells lysed, and equal cell equivalents of cell lysates exposed to an excess of Tf-agarose. The agarose was collected, washed, and bound lysate proteins were removed and separated by SDS-PAGE. After western blotting of bound proteins, the biotin groups were detected by exposure of the blot to streptavidin-HRP followed by ECL. Left: results of the ECL; right: densitometric scans of the lanes shown on the left. The ratio of the areas under the TfR peaks was 5.8:1 (MTLn2-Tf20 : MTLn2).

Fig. 6. Scatchard analysis of transferrin binding to MTLn2 and MTLn2-Tf20 cells. The cells were grown to 70-80% confluence on 12-well plates, washed twice with serum-free medium, and equilibrated at 4°C. Increasing amounts of 125 I-transferrin were added to wells in replicates of five. Two hours later, the cells were washed and lysed, and cell bound (lysate) radioactivity was determined using a gamma counter. To control for non-specific binding, two wells of each dose received a 200-fold excess of unlabeled transferrin. Nonspecific bound radioactivity obtained from these wells was then subtracted from the data. Results are representative of two experiments.

Fig. 7. Photographs of representative lungs from rats injected with the MTLn2 parental cell line (top) or the MTLn2-Tf20 cell line (bottom).

Table 1. Results of spontaneous metastasis assays. Syngeneic female Fisher 344 rats were injected with 1×10^6 MTLn2-Tf10, or MTLn2-Tf20 cells into the left mammary fat pad. Six weeks later, rats were sacrificed and examined for gross inguinal, axillary, and lung metastasis. All measurements are in millimeters.

Cell Line	1° tumor median diameter	# with axillary node metastases/ total number injected	median diameter of lymph node metastases	# with lung metastases/ # injected	median # of lung metastases	range of lung metastases
Experiment #1.						
MTLn2	13	0/9	0	0/9	0	0
MTLn2-Tf20	16.5	6/10	5	6/10	18	0->100
Experiment #2						
MTLn2	14	1/6	0	0/6	0	0
MTLn2-Tf10	10	3/6	3	1/6	0	0->100

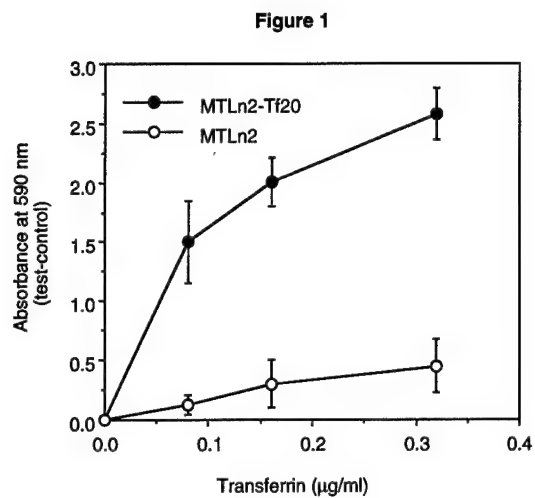


Figure 3

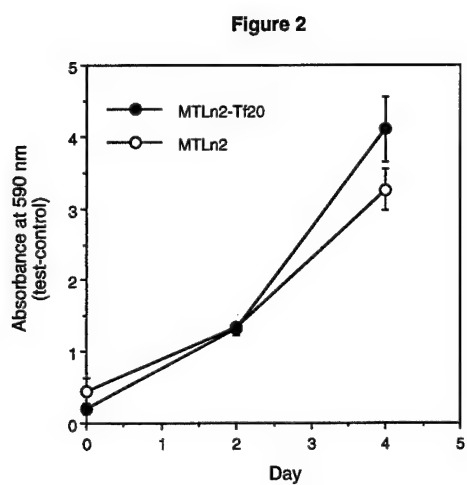
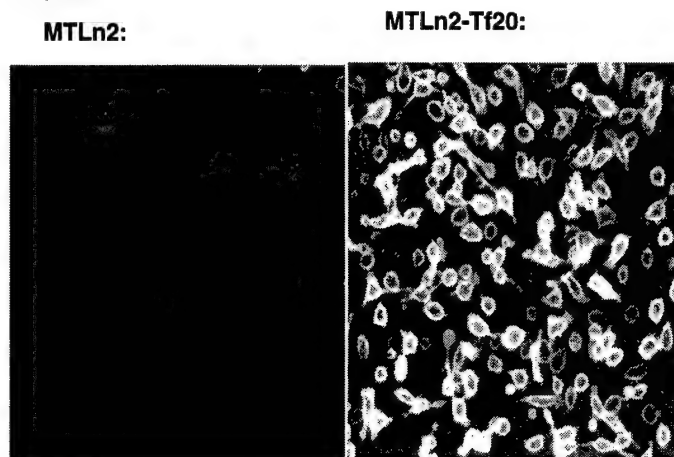


Figure 4

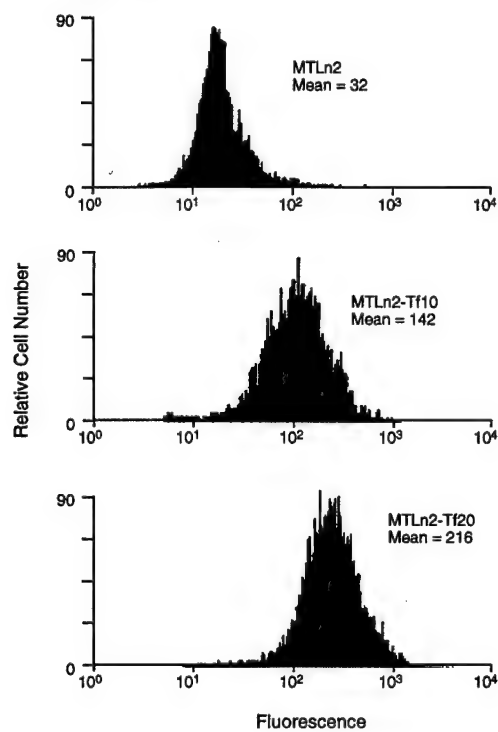


Figure 5

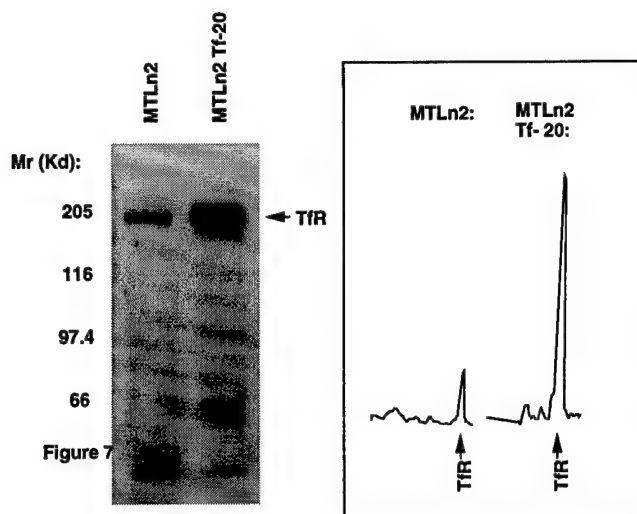


Figure 6

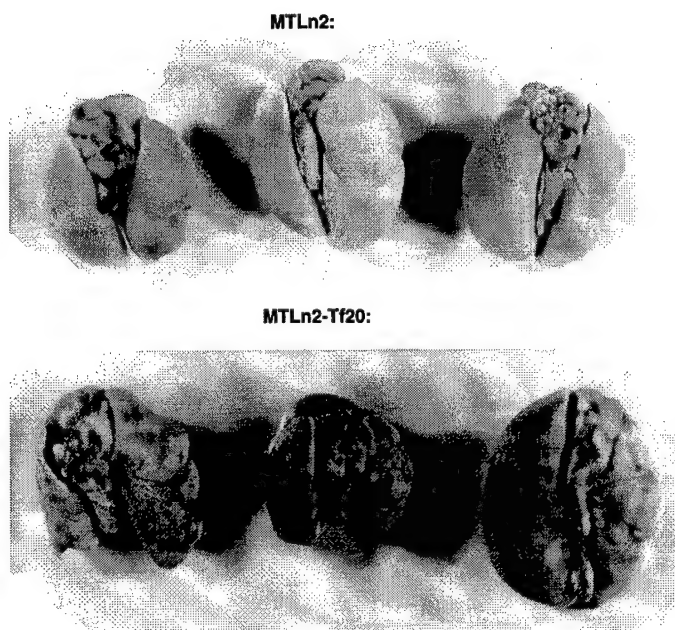
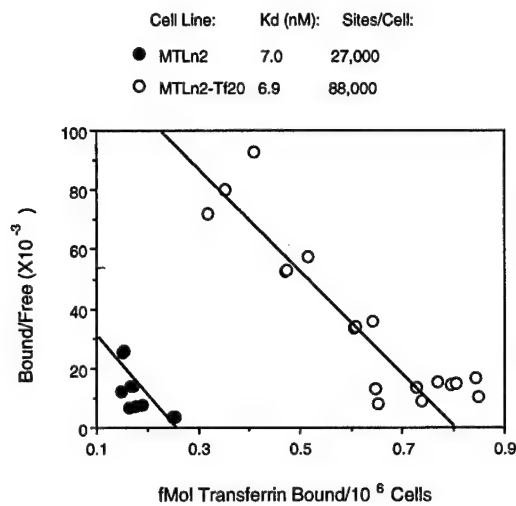


Figure 7

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 Reprint #1 Selection of highly metastatic...

**Rat MTLn2 mammary adenocarcinoma cells transfected with the transferrin receptor gene
exhibit an enhanced response to transferrin and an increased metastatic capability.**

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Running Title: Tumor cell transferrin responsiveness in metastasis.

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Summary

We previously found that breast cancer cell transferrin receptor expression and proliferative response to transferrin often correlated with metastatic capability. To further explore this, we transfected tumor cells with the cDNA coding for the transferrin receptor and examined the effects of its over-expression on various cellular properties. A human transferrin receptor expression plasmid was made by excising the cDNA for the receptor from pcDTR1 and ligating it into the multiple cloning site of pcDNA1Neo. The rat MTLn2 line was initially transfected with this construct, since it is poorly metastatic, had been found by us to express low endogenous levels of rat transferrin receptor, and being non-human, presented a system where background human receptor expression was nonexistent, therefore, transfection-induced receptor expression could be ascertained using antibodies specific for the human protein. Approximately 50% of the initial Geneticin-resistant transfected MTLn2 cells over-expressed human transferrin receptor protein. High expressors were further isolated by four sequential FACs sorts. The final population expressed $\approx 7 - 12$ times more cell surface transferrin receptor than did vector transfected controls. Both lines proliferated at the same rate in normal (media plus 5% FBS) culture conditions. However, in serum-free conditions, the transferrin receptor over-expressors displayed a pronounced proliferative response to transferrin whereas the control line did not. When injected into the mammary fat pad of female nude mice, cells from both lines formed micrometastases to the lung, which could be specifically visualized by immunohistochemical staining of rat cytokeratin 17. This revealed that the transferrin receptor transfected line formed larger, more frequent lesions of this nature than did cells from the vector transfected controls. Over expression of cell surface human transferrin receptor on MTLn2 cells appeared to effect their *in vitro* growth response to transferrin and their ability to form metastases *in vivo*. Key Words: Transferrin, Transferrin Receptor, Metastasis, Breast neoplasms.

Introduction

Tumor cell response to normal organ environment-situated factors which regulate growth (1 - 3), motility (2 - 5), adhesion (2,3,6), and other cellular responses (2,3) have been shown to be involved in successful metastases to a particular target. In exploring one aspect of target organ environmental effect on tumor cells, we examined the ability of media conditioned by viable organ fragments to influence the *in vitro* proliferation of cells which either possess or lack a propensity to metastasize to that organ site (7 - 10). In this regard, we found that lung conditioned media caused a preferential growth of certain lung-metastasizing tumor cells when compared to cells which do not metastasize to that location (7 - 10). In particular, the lung metastasizing MTLn3 derivative of the 13762NF rat mammary adenocarcinoma was found to exhibit a proliferative response to rat lung conditioned medium whereas non-metastatic MTPa, and MTC cells derived from the same tumor system did not (9,10). Traditional biochemical means were utilized to purify the MTLn3 mitogen from the lung conditioned media and this proliferation-enhancing factor was eventually identified as the iron transport protein transferrin (9 - 11). Others have also observed that tumor cell proliferative response to transferrin correlates with metastatic capability and that transferrin-responsiveness may be involved in secondary site-specific tumor cell growth (12 - 14).

Transferrin exerts a proliferative effect on cells in culture by supplying iron for key growth-dependent synthesis processes. Transferrin binds two atoms of iron, one at each of two sites located at the amino terminal and carboxy terminal domains of the protein. Iron replete transferrin interacts with a cell surface transferrin receptor (TfR), a homodimeric disulfide-linked glycoprotein of $M_r \approx 190,000$. The receptor-ligand complex is internalized in structures which mature into acidic endosomes; the low pH of which results in the release of the bound iron from the complex (15,16). Iron is then translocated to needed areas such as sites of synthesis of mitochondrial electron transport proteins and ribonucleotide reductase (15, 16). The latter enzyme consists of two non-identical R1 and R2 subunits, of which the iron saturated state of R2 is required for activity. Maintenance of the function of this enzyme is essential for the synthesis of DNA and the proliferation of cells (15 - 17).

Observation of a preferential response to transferrin in certain cell lines of the rat mammary adenocarcinoma system led us to study the TfR expression of those same cells. Scatchard analysis of transferrin binding by various 13762NF sublines revealed that TfR expression also correlated with metastatic capability (18). Others have also found that TfR expression in neoplasms associates with tumor stage, progression, or predicted survival. In a series of immunohistological studies, a

correlation of this nature has been seen in melanomas (19,20), breast carcinomas (21,22), bladder cell transitional cell carcinomas (23), a maxillary cancer (24), and in nonsmall cell lung cancer (25).

The indicated connection between metastatic capability, transferrin responsiveness, and TfR expression in certain tumor cells lent us to examine as to whether or not we could affect the metastatic capability of one of these cell lines by altering these latter characteristics. In this regard, we found that a highly transferrin-responsive subline culled from the low transferrin-responsive, low metastatic rat MTLn2 13762NF mammary adenocarcinoma subline did indeed express higher TfR numbers and exhibited an enhanced metastatic capability in syngeneic Fisher 344 rats (26, 27). These observations influenced us to pursue a more direct approach to altering tumor cell TfR expression, one which would not involve a prolonged *in vitro* selection processes that had also potentially selected for other cell properties. The transfection of a cell line with a plasmid encoding the sense TfR cDNA was a logical approach to this, with the hope that the only cell trait altered would be that of TfR expression. A human system was the model of choice since the only complete TfR cDNA available was that encoding the human transferrin receptor (hTfR). However, initial success with the hTfR construct was dubious and a system which displayed clear-cut evidence of successful plasmid-induced hTfR expression was desired. This was achieved using a non-human cell line as a recipient for transfection, thus specific detection of expressed protein could be performed using antibodies particular for the human receptor. In our hands, the MTLn2 cell line expressed relatively low amounts of rat TfR protein, exhibited a meager proliferative response to transferrin, and displayed a poor metastatic capability in syngeneic rats. As such, the line was a convenient indicator of plasmid effectiveness, and one which was advantageous for examining effects of TfR over-expression. Thus, the line was chosen as a transfection subject.

In this study, we report on the construction of a eukaryotic expression plasmid containing the human hTfR cDNA. We found that transfection of human MTLn2 mammary adenocarcinoma cells with this construct was shown to be able to increase cell surface hTfR expression. When compared to vector transfected controls, the hTfR-transfected cells were observed to proliferate more rapidly in response to transferrin serum free conditions. This observation led us to perform spontaneous metastases assays in nude mice, where we found that the hTfR-transfected line were also able to form greater numbers of metastatic lesions than did vector transfected control cells.

Materials and Methods:

Cells and Cell Culture. The rat 13762NF mammary adenocarcinoma was originally developed in Fischer 344 rats by dietary administration of 7,12-dimethylbenz[a]-anthracene; the MTLn2 line was originally cloned from a lung metastasis arising from a 13762NF tumor growing in the mammary fat pad (28). The line displayed little spontaneous or experimental metastatic ability in syngeneic F344 rats (28,29). The MTLn2 line was maintained at 37°C in a humidified 95% air - 5% CO₂ atmosphere in α -MEM containing 5% (vol/vol) fetal bovine serum (FBS, Hyclone, Logan, UT). The line was passaged by removal from plates using PBS containing 0.25% trypsin (Gibco/BRL, Bethesda, MD) and 2 mM EDTA, and was routinely examined and found to be free of mycoplasma.

Plasmid construct. The pcDTR1 plasmid containing the entire coding region of human TfR was obtained from Dr. Lukas Kuhn of the Swiss Institute for Experimental Cancer Research (Epalinges, Switzerland). The plasmid was cut with EcoRV and XbaI and the 2.3 kb fragment containing the TfR coding region (minus the 3' un-translated region) was isolated by agarose gel electrophoresis and electroelution. The pcDNA1/Neo eukaryotic expression plasmid (Invitrogen, San Diego, CA) was treated with the same two enzymes, and the 7.4 kb linearized plasmid likewise isolated. The TfR fragment was ligated into pcDNA1Neo by conventional procedures using T4 DNA ligase at 16°C. Bacteria were transformed with the ligation product and plasmid DNA minipreps obtained from ampicillin resistant colonies were analyzed for their ability to release a 2.3 kb fragment upon digestion with EcoRV and XbaI. One colony expressing this plasmid was expanded and plasmid DNA to be used for transfection procedures was isolated using a Qiagen maxi-kit (Qiagen, Chatsworth, CA).

Transfection procedure. MTLn2 cells were grown to 40 - 50 % confluence in six-well culture plates. Cells were transfected for 8 h using 0.2 - 0.5 ng of plasmid DNA per well in serum-free media containing 5% vol/vol Lipofectamine (Gibco). Lipofectamine-media-DNA mixtures were made as per the manufacturer's suggested protocol. Media was replaced with that containing 5 % FBS for 24 h

and then with media containing 5% FBS and 400 $\mu\text{g/ml}$ G418 (Geneticin, Gibco). Cells resistant to G418 were passaged and maintained in media containing 5% FBS and 400 $\mu\text{g/ml}$ G418.

Immunofluorescent Detection of Cell Surface TfR. Cells grown on LabTek (Nunc, Naperville, IL) slides were washed 3 times with 25 mM HEPES (pH 7.5) buffered Dulbecco's-modified minimal essential medium containing 1 mg/ml liquid gelatin (Sigma, St. Louis, MO; DMEM-Ig) and equilibrated to 4° C. Phycoerythrin-conjugated anti-human TfR (clone T56/14; Biodesign International, Kennebunkport, ME) or phycoerythrin-conjugated normal mouse IgG (Biodesign International) were diluted 1:100 in 4° C DMEM-Ig, were added to the cells, and the slides were incubated on ice for 2h. The cells were washed 3 times with DMEM-Ig and examined for fluorescence using a Nikon Diaphot phase contrast microscope (Melville, NY). The procedure utilized for staining for the rat TfR was identical with the exception that the cells were initially incubated with mouse anti rat-TfR (clone OX-26, BPS, Indianapolis, IN), or normal mouse IgG₁, then with an Fab' fragment of PE-conjugated anti-mouse IgG (Zymed, San Diego, CA).

FACS analyses and sorting. All washes and antibody incubations were performed using DMEM-Ig. Cells were removed from culture plates using 0.25% trypsin, 2 mM EDTA (in Ca²⁺, Mg²⁺-free PBS). As soon as detachment was complete, FBS was added to 1% (v/v), to neutralize the trypsin. Cells were washed twice by centrifugation and resuspension in DMEM-Ig, counted, cell density was adjusted to $1 \times 10^6/\text{ml}$, and the cell suspensions were equilibrated to 4° C. Phycoerythrin-conjugated normal mouse IgG or anti-human-TfR was added to the suspensions to a final dilution of 1:100, the cells were incubated at 4° C for 2 h, washed twice with DMEM-Ig, and were analyzed for fluorescence using a Becton-Dickinson FACscan instrument (San Jose, CA). Identical procedures were used for sorting experiments, with the exception that cells were analyzed and those cells exhibiting the maximum 5% fluorescence in the anti-TfR group were sorted using a Becton-Dickinson FACstar instrument.

Affinity Isolation of TfR using Immobilized transferrin. This method was adapted from TfR isolation procedures reported by others (30,31) and was performed as previously described (27). Human transferrin (apo form) was immobilized onto cyanogen bromide activated agarose, and iron saturated by exposure to ferric ammonium citrate. Cells were surface biotinylated at 4° C using NHS-LC-biotin, were lysed, and equal amounts of lysate protein added to an excess of the transferrin-agarose. The suspensions were mixed for 2 h at 4° C, the agarose was collected, washed, and bound material was removed with SDS-PAGE treatment solution. Released components were separated by SDS-PAGE, blotted onto a PVDF membrane, and biotinylated bands were detected by incubation with streptavidin conjugated HRP (horse radish peroxidase) followed by an ECL (enhanced chemiluminescence) HRP substrate (DuPont, Wilmington, DE). Bands were quantitated using a Hoeffer model GS300 scanning densitometer (San Francisco, CA).

Scatchard analysis of cell surface TfR. This was performed as previously described (27): Human holo-transferrin was radiiodinated using the chloramine-T method. Cells at 50 - 60% confluency growing in 12 well plates were rendered transferrin deficient, equilibrated to 4° C, and were treated with increasing levels (5 wells per level) of ¹²⁵I-Tf. Two wells of each dose also received a 200-fold excess of unlabeled Tf. Cells were incubated at 4° C for 2h, washed, lysed, and lysate radioactivity was determined. Specific bound counts were calculated and data was plotted.

Rhodamine-Transferrin Uptake. Cells were grown on multi-chambered LabTek slides to 40-50 % confluence. Cells were washed twice for 1 h each in α -MEM only. Media was changed to DMEM-Ig and rhodamine conjugated human transferrin was added to a final concentration of 0.5 $\mu\text{g/ml}$. Negative control wells also received un-conjugated transferrin at a concentration of 20 $\mu\text{g/ml}$. Two hours later, wells were washed three times with DMEM-Ig, equilibrated to 4° C, and cells were examined for fluorescence.

Cell growth assays. Cells were made transferrin deficient by placement in 2 24h changes of serum-free α -MEM. Cells were removed from stock plates and seeded in 100 μL α -MEM containing 2 mg/ml bovine serum albumin (BSA) at a density of 2,000 cells/well in 96 well plates. One day later, cells were washed twice with and the medium was changed to 100 μL DME/F12 containing 0.0% FBS, and increasing amounts of holo-human transferrin was added into test wells. Five days later, the cells

were quantitated using a crystal violet stain assay (27): Cells were washed with PBS, were fixed at 25°C for 30 min with PBS containing 5% vol/vol glutaraldehyde, were washed with water, allowed to dry, and were stained for 30 min at 25°C with 50 μ L 0.1% crystal violet in 50 mM CAPS, pH 9.5. Stained cells were washed with water and were solubilized with 50 μ L 10% acetic acid. Cell numbers were determined by measuring Absorbance at 590 nm on a Dynatech (Chantilly, VA) model MR5000 plate reader. Absorbance in this system directly correlates with cell number up to \approx 50,000 cells/well (10, 11, 32).

Spontaneous Metastasis Assays. All experimentation involving animals has been approved by the Institutional Animal Care and Use Committee. All animals are treated and housed under conditions specified by the NIH, the Department of Health and Human Services and the Department of Agriculture. Cells at 50-70% confluence were removed from culture plates with Ca^{2+} , Mg^{2+} - free Hank's basic salt solution (CMFH) containing 1 mM EDTA and 0.25% trypsin. Cells were washed 3 times with CMFH, cell density was measured using a model ZM Coulter Counter and adjusted to 5×10^6 /ml with CMFH. Female nu/nu mice (age: 6 - 8 weeks) were anesthetized with Metofane (Methoxyflurane; Pittman-Moore, Washington Crossing, NJ). A small incision was made \approx 1.0 cm posterior to the left fore leg and 0.1 ml of the cell suspension was injected into the left mammary fat pad lateral to the rib cage \approx 0.5 cm posterior to the fore leg (33,34). The incision was closed with a wound clip. Four-six weeks later, animals were killed with an overdose of Metofane and were examined for the presence of metastatic lesions.

Immunohistochemical staining for cytokeratin-17: Lungs were removed from nude mice, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Blocks were sectioned at 5 μ m thickness and resulting slides stored at 25°C. Sections were de-paraffinized in two changes of xylene, equilibrated in two changes of absolute ethanol, treated with 10% H_2O_2 (in methanol) for 30 min., placed in 10 mM citric acid (pH 6.0), and exposed to 5 min heating in a microwave oven. Sections were washed in PBS and incubated in a block solution (PBS containing 5% v/v goat serum and 2 mg/ml gelatin) for 30 min. All subsequent antibody incubations were performed in block solution and washes in PBS occurred between all steps. Slides were incubated overnight at 4°C in 1:100 normal mouse IgG₁ or anti Cytokeratin-17 (Chemicon, Temecula, CA), for 2 h at 25°C in 1:100 biotin-conjugated anti-mouse IgG (Sigma), and for 1 h at 25°C in 1:100 streptavidin-HRP (Sigma; 25°C, 1h). Slides were incubated in DAB substrate (Sigma) for 5 min., washed, equilibrated in absolute ethanol followed by xylene, coverslipped, and examined by phase contrast microscopy. The size of metastases seen in each line were compared using the Kruskal-Wallis test.

Results.

A schematic displaying the eukaryotic expression construct made containing the entire coding region for human TfR is shown in Figure 1. The MTLn2 cell line exhibited excellent transfection properties with this plasmid: cells transfected with both the vector (MTLn2/Neo) and hTfR plasmid (MTLn2/hTfR) formed confluent wells of G418 resistant cells. Therefore, no initial cloning or colony selection was required; analysis of hTfR expression was performed on uncloned initial transfected populations. Immunofluorescent analysis of these by FACS indicated that the vector transfected cells expressed no detectable hTfR protein (Figure 2). In contrast, approximately 50% of the hTfR transfected cell population displayed fluorescence over the isotype stained control, indicating hTfR expression and successful transfection (Figure 2). Those MTLn2/hTfR cells exhibiting the highest 5% fluorescence were sorted, re-cultured, likewise stained, and sorted again. This process was repeated four times, and during the sorting process, the mean hTfR expression was seen to increase slightly (Figure 3). The heterogeneity in hTfR expression observed in MTLn2/hTfR cells was not due to variation caused by analyzing cells in various phases of the cell cycle, as synchronization of the cells in the G1-S phase by aphidicolin had no effect on the FACS signal seen (data not shown). Fluorescent microscopic examination of MTLn2/hTfR cells stained unfixed at 4°C with PE-anti-hTfR revealed cell surface fluorescence of varying intensity in nearly every cell, with no visible fluorescence observed in like-treated MTLn2/Neo cells (Figure 4). This difference in hTfR expression was stable over many (5-10) passages in cultures maintained in G418 containing media (data not shown). Both lines stained positive for the rat TfR, albeit a lower level of signal was obtained for this when compared to hTfR (figure 4).

Analysis of cell surface hTfR content in these cell lines by affinity isolation of biotinylated TfR revealed that the MTLn2/hTfR line expressed 7-8 fold more total TfR than the MTLn2/Neo (Figure. 5). Scatchard analysis of ¹²⁵I-transferrin binding to MTLn2/Neo or MTLn2/hTfR cultured cells at 4°C showed a much higher specific binding for the MTLn2/hTfR line, again indicating greater numbers of TfR on these cells (figure 6).

Visual fluorescence examination of MTLn2/Neo and MTLn2/hTfR cells allowed to take up rhodamine conjugated transferrin indicated a much stronger fluorescent signal for the MTLn2/hTfR line (Figure 7). This indicated that the increased TfR in this line was functional and, that over the 24h incubation time, much more transferrin was internalized by the MTLn2/hTfR than by the MTLn2/Neo line.

Once demonstration of a remarkable, stable increase in functional TfR expression in the MTLn2/hTfR transfected cells was ensured, attempts to measure an increase in that line's ability to proliferate in response to transferrin were made. In these assays, cells are plated overnight at low density in media containing 1% FBS, media is replaced with that containing no serum, increasing amounts of human holo-transferrin are added, and cell numbers are determined four days after transferrin addition (9,10,27). Here, we found significant background growth (in the 0 µg/ml transferrin wells) with the MTLn2/hTfR line. It was thought that this line had become hypersensitive to transferrin, and was proliferating in the serum free/transferrin-free environment in response to residual transferrin remaining from the 1% FBS plating conditions. Therefore, the assay was repeated with an effort to more thoroughly eliminate serum from the cells. Stock cultures were exposed to two 24h rounds of serum-free incubations, then subcultured into the assay plate, also in serum-free media. These severe conditions eliminated background proliferation in the MTLn2/hTfR cells, and when transferrin was added into this system, a proliferative effect was seen for this line, but not for the MTLn2/Neo cells (Figure 8). The results indicated that the MTLn2 line had become hyper-responsive to transferrin, and that the addition of this protein permitted the proliferation of this line in serum-free conditions.

The finding of an increased proliferative rate for the MTLn2/hTfR cells in serum deprived conditions lent us to investigate as to whether or not the metastatic properties of the line had changed. Nude mice were chosen as recipient animals, since the transfected gene expressed was human, thus it was feared that an *in vivo* test in syngeneic F344 rats would result in immune system-mediated rejection of the tumors. It was also desired to perform spontaneous metastasis assays, since these are more rigorous than experimental assays, and necessitate complete metastatic competence in the cells being studied. To ensure injection into the proper primary site, the cut-down procedure of Price *et al.* (34) was used. Using this method, cells are injected into a mammary fat pad adjacent to the rib cage. This was desirable, since we had found that placement of the cells into an abdominal mammary fat pad (without a cut down procedure) resulted in the frequent formation of intra-peritoneal metastases, which were of doubtful quality. After injection into the left mammary fat pad of female nude mice, it was found that either line formed 1° tumors of approximately the same size at approximately the same rate (Table 1). The spontaneous metastases test was terminated at five weeks as some of the primary tumors approached the maximum size allowable. Necropsy examination revealed no gross metastases in the nude mice injected with either line, therefore, lungs from recipient animals were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin, and examined for micrometastases by light microscopy. Serial sections from two separate areas of lung tissue were examined. This revealed 2 small (100 - 200 µm diameter) micrometastases in MTLn2/Neo injected mice and 6 larger (2000 - 5000 µm diameter) like lesions in MTLn2/hTfR treated animals. Immunohistochemical staining of the sections with anti-rat cytokeratin 17 enabled for a more definitive and sensitive micrometastasis detection. Use of this procedure revealed additional lung tumors which were enumerated and shown in table 1. The table reveals that either line formed meager numbers of micrometastases, however, it was clear that the MTLn2/hTfR line was the more metastatic of the two, forming increased numbers of metastatic lesions of increased size ($p < 0.05$), resulting in a total lung tumor burden of approximately seven times that of the MTLn2/Neo line. Representative micrographs of lung micrometastases seen are shown in Figure 9.

Cultures of MTLn2/hTfR micrometastases were established from lung fragments taken from two of the mice injected with this line. When analyzed for hTfR expression by immunofluorescence, these cells stained positive with an intensity equal to that of the original injected MTLn2/hTfR cells,

indicating that hTfR expression was maintained throughout the process of *in vivo* growth and metastasis formation.

Discussion:

We had previously found a correlation between tumor cell TfR-dependent proliferative response to transferrin and the metastatic behavior of certain cell lines. The observation of a tumor cell response associated with high metastatic ability has led many investigators to strengthen that observation by increasing the expression of cellular constituent(s) necessary for that response via transfection. If an enhancement of metastatic behavior was seen, then a more positive statement about the role of that system in metastasis could be made. In this regard, others have reported on the augmentation of metastatic behavior of low metastatic cells by the transfection of those cells with a plasmid carrying the cDNA for a purported metastasis-associated factor (35-42). Likewise, we desired to add credence to our previous observations by modifying and increasing the metastatic capability of one or more low metastatic tumor cell types by transfecting them with a plasmid carrying the gene encoding the TfR.

The initial step in assessing the performance of the hTfR plasmid construct we had made was to examine its ability to cause increased human TfR expression in a test line. The MTLn2 line was chosen for this, since we found it to express no immuno-detectable human TfR in typical culture conditions, therefore, it afforded a system where the presence of transfection-dependent protein could be made in the absence of background signal. Also, since this was a previously defined low-transferrin responsive, poorly-metastatic rat breast cancer line (26, 27), it posed as one where the effects of TfR over-expression might be more clearly elucidated. It was to our surprise to find that the MTLn2/hTfR transfected cells had acquired the ability to proliferate in response to transferrin in severe serum-starved conditions. This gave us incentive to perform metastasis studies in nude mice where we found that the MTLn2/hTfR transfectants appeared to gain an increased competence to form lung micro-metastases. The targeting to the lung of the cells used in the present study was probably due to the previously established behavior of this line. Increased TfR expression most likely did not bring about lung targeting, but may have caused increased growth of the metastatic lesions at that site, as high levels of transferrin have been reported to occur in adult perfused lung tissue (12, 46).

The relative rarity of metastases seen made it desirable to assess their presence using a technique more sensitive than hematoxylin and eosin staining. The obvious choice to immunohistochemically stain lung sections for rat and human TfR was considered, however, this would have entailed the use of frozen sections and antibodies of unknown cross-reactivity to mouse TfR. An anti-rat and human cytokeratin-17 antibody was found that could specifically stain the rat tumor cells used, with minimal or no staining of murine lung cells. The capability of this antibody to perform in formalin-fixed paraffin embedded tissues further supported its use as the reagent of choice for micrometastasis detection. Metastases enumerated with this reagent clearly indicated an increased metastatic ability for the MTLn2/hTfR line.

The results obtained here indicate that a more pronounced difference between the two lines in regard to metastasis formation might be seen if the *in vivo* experiment could be prolonged. To do this, efforts to repeat the experiment with the inclusion of primary tumor excision 2 - 3 weeks after injection stage are now underway.

As mentioned earlier, a proposed role for transferrin responsiveness and transferrin receptor expression in metastasis has been made by many other investigators (12-14, 19 - 24). However, to our knowledge, no others have reported on the manipulation of TfR expression or transferrin response in an attempt to influence the metastatic performance of a certain tumor cell type. Sasaki *et. al.* (43) have reported that the transfection of a human hepatoma cell line with an antisense TfR construct can reduce cell surface TfR numbers, however, no mention is made of any effect this had on tumorigenicity or metastatic capability of those cells. The TfR construct we used lacks the 3' untranslated region, which contains sites through which TfR expression is regulated, via recognition by iron regulatory site binding proteins (44). Others have observed that transfection with a similar plasmid produces cells with increased sensitivity towards H₂O₂ mediated DNA damage (44). However, we did not observe any obvious increase in the fragility of our transfected cells, which indicates that this type response was not apparent.

The assessment of the metastatic capability of a rat tumor line in nude mice is somewhat unusual, however, this was necessitated by the presence of a human protein on the cell's surface. However, this type of experiment has been performed occasionally, an example being the testing of the metastatic capability in nude mice, of a rat mammary adenocarcinoma line transfected with the human epidermal growth factor receptor (45).

One of the aspects of these findings which requires further study is the mechanism of tumor cell response to transferrin and an elucidation of as to whether or not this is through traditional iron delivery-dependent mechanisms or through more exotic TfR-mediated signal transduction events similar to those seen in T cells (47). A clearer understanding of this response should aid in the knowledge of metastatic behavior.

These results confirmed previous observations and indicated that breast tumor cell expression of TfR can be an important characteristic for metastasis formation, at least in some tumor-cell systems. The results suggest that continued exploration of therapeutic strategies that interfere with transferrin binding or iron uptake by tumor cells (48,49) are warranted.

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Table 1: Results of spontaneous metastases assays.

Mouse #	1° Tumor Volume (cm³):	Total # of Micrometastases:	Diameter of Micrometastases (μM):
MTLn2/Neo:			
1	1.52	0	
2	0.48	3	200, 200, 300
3	1.26	2	40, 300
4	1.26	1	100
5	2.85	1	100
6	2.45	2	300, 300
7	0.76	2	400, 400
8	0.10	0	
9	1.02	0	
(total)		11	2940
average	0.77	1.22	327
MTLn2/TfR:			
1	0.34	1	200
2	0.34	1	100
3	1.13	1	40
4	0.72	2	2500, 4500
5	0.12	5	20, 400, 1600, 2500, 2500
6	0.53	0	
7	0.94	3	200, 200, 400
8	0.53	2	120, 1200
9	0.53	3	200, 400, 600
10	1.41	2	1200, 2500
(total)		20	21380
average	0.66	2.00	2138

Table 1. Results of spontaneous metastases assays. Female nude mice were injected into the left mammary fat pad with 5×10^5 MTLn2/Neo or MTLn2/TfR cells. Five weeks later, mice were sacrificed, lungs were fixed in formalin, embedded in paraffin, and sectioned. Lung sections were stained immunohistochemically using an anti-rat cytokeratin-17 antibody and specifically stained rat tumor micrometastases were enumerated. The results indicate relatively few metastases for either group, but that larger, more frequent micrometastases were formed by the MTLn2/TfR cells than by the MTLn2/Neo cells.

Figure Legends

Figure 1. A: schematic illustration of the formation of the TfR coding construct where the human TfR coding region was removed from pcDTR1 and cloned into pcDNA1Neo. **B:** Results of agarose gel analysis of endonuclease cut pcDTR1 and pcDNA1Neo/TfR. Lane 1: HindIII digested lambda DNA standards; Lane 2: the results of digestion of pcDTR1 with EcoRV and XbaI. The middle (2.5 kb) fragment containing the TfR coding region was cloned into pcDNA1Neo (7.1 kb). The resulting plasmid displayed a 9.6 kb band when linearized with Eco RV (lane 3), and two fragments of 7.1 and 2.5 kb when treated with EcoRV and XbaI (lane 4).

Figure 2. Immunofluorescent analysis of TfR expression on MTLn2/Neo and MTLn2/hTfR cells by FACS. Cells were removed from plates, stained at 4°C with either a PE-conjugated anti-human TfR or PE-conjugated mouse IgG₁ and analyzed. **A:** MTLn2/Neo cells treated with PE-conjugated mouse IgG₁ (MTLn2/hTfR cells displayed an identical pattern; data not shown); **B:** MTLn2/Neo cells stained with PE-conjugated anti-human TfR, **C:** MTLn2/TfR cells stained with PE-conjugated anti-human TfR. Approximately 50% of the MTLn2/hTfR transfected population displayed immuno-detectable human TfR.

Figure 3. Immunofluorescent analysis of TfR expression on FACS sorted MTLn2/TfR cells. Cells were removed from plates, stained at 4°C with PE-conjugated anti-human TfR or mouse IgG₁ and analyzed. A slight increase in TfR expression was evident as sorting progressed from the first sort (MTLn2/hTfR FACS1) to the fourth sort (MTLn2/hTfR FACS4).

Figure 4. Representative fluorescent micrographs from MTLn2/Neo and MTLn2/hTfR cells stained unfixed at 4°C with PE-conjugated anti-human TfR, or anti-rat TfR followed by PE-conjugated anti-mouse IgG. **A:** MTLn2/Neo cells as seen with visible light; **B:** UV-light illuminated photograph of cells in A stained with anti-rat TfR (MTLn2/hTfR cells appeared identical to A and B when like-treated); **C:** UV-light illuminated photograph of MTLn2/Neo cells stained with anti-human TfR. **D:** UV-light illuminated photograph of MTLn2/hTfR cells stained with anti-human TfR. When either cell line was treated with PE-conjugated normal mouse IgG₁, no fluorescent signal was obtained (data not shown).

Figure 5. Results from measurement of cell surface MTLn2/Neo or MTLn2/hTfR by affinity isolation of biotinylated TfR. Cells were made transferrin deficient, surface biotinylated at 4°C, lysed, and equal lysate cell equivalents incubated with an excess of transferrin-agarose. The agarose was washed, bound material removed, separated by SDS-PAGE, and blotted onto a PVDF membrane. Biotinylated bands were detected by incubation with streptavidin-HRP and exposure to an ECL HRP substrate. The ratio of MTLn2/hTfR : MTLn2/Neo TfR was 6.3:1. **A:** results of ECL, **B:** results of densitometric scan of A.

Figure 6. Scatchard analysis of ¹²⁵I-transferrin binding to MTLn2/Neo and MTLn2/hTfR cells. Cells were grown on 12 well plates, made transferrin deficient, and incubated at 4°C with increasing levels of ¹²⁵I-transferrin. Negative control wells also received a 200-fold excess of unlabeled transferrin. Cells were incubated for 2h at 4°C, washed, lysed, and radioactivity determined. Points represent the mean ± SD of three wells corrected for non-specific binding.

Figure 7. Representative fluorescent micrographs of MTLn2/Neo and MTLn2/TfR cells allowed to internalize rhodamine-conjugated transferrin. Cells were grown on multi-chambered slides, made transferrin-deficient, incubated at 37°C for 24h with 0.5 µg/ml rhodamine-conjugated transferrin, washed, and analyzed by fluorescent microscopy. **A:** results from the MTLn2/Neo line; **B:** results from the MTLn2/hTfR line. No fluorescence was seen when a 50-fold excess of un-conjugated transferrin was included (data not shown).

Figure 8. Results of the analysis of the proliferative response of MTLn2/Neo and MTLn2/hTfR cells to transferrin. Cells were plated at 2,000 cells/well in 96 well plates in serum-free media. One day later, cells were washed, and serum-free media with increasing levels of transferrin was placed into wells. Five days later, cells were quantitated using a crystal violet stain assay. **A:** results of

absorbances obtained from the crystal violet stain assay; **B**: appearance of the stained assay plates prior to dye solubilization. Points represent the mean \pm SD from four wells.

Figure 9. Representative micrographs of MTLn2/Neo and MTLn2/hTfR tumors growing in nude mice. **A**: section of an MTLn2/Neo primary mammary fat pad tumor; the center of which is indicated by the arrow. **B**: a typical MTLn2/Neo lung micrometastasis, indicated by the arrow. **C**: a typical MTLn2/hTfR lung micrometastasis; arrows indicated the borders of the tumor. **D**: an MTLn2/Neo lung micrometastasis stained with anti-cytokeratin-17, indicated by the arrow.

Figure 1

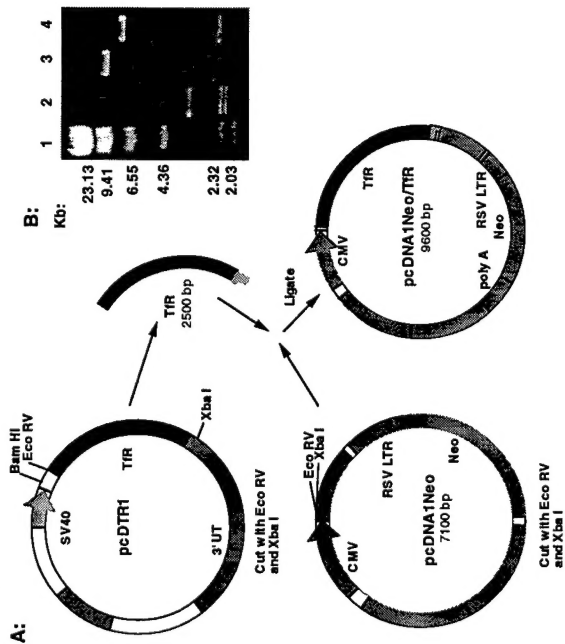


Figure 2

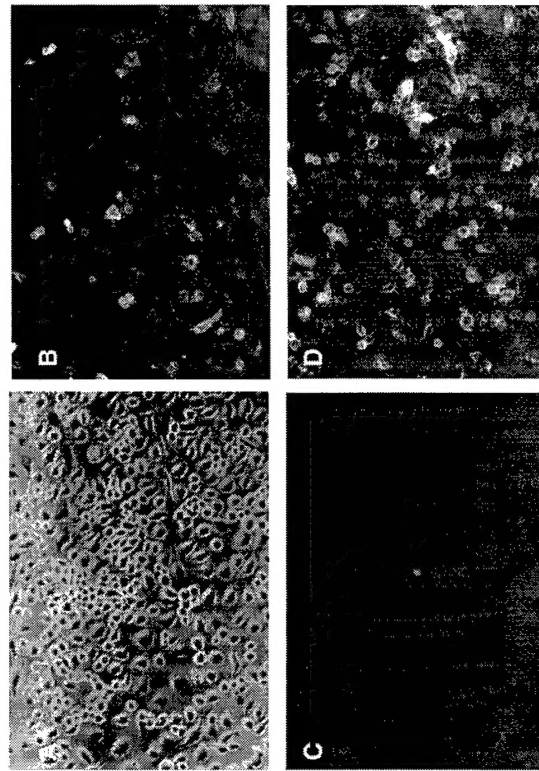
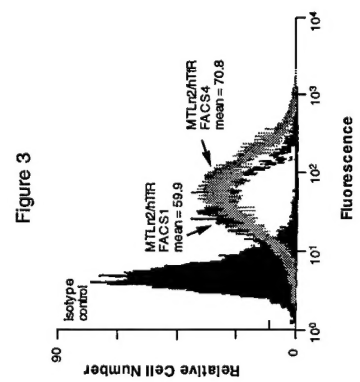


Figure 3



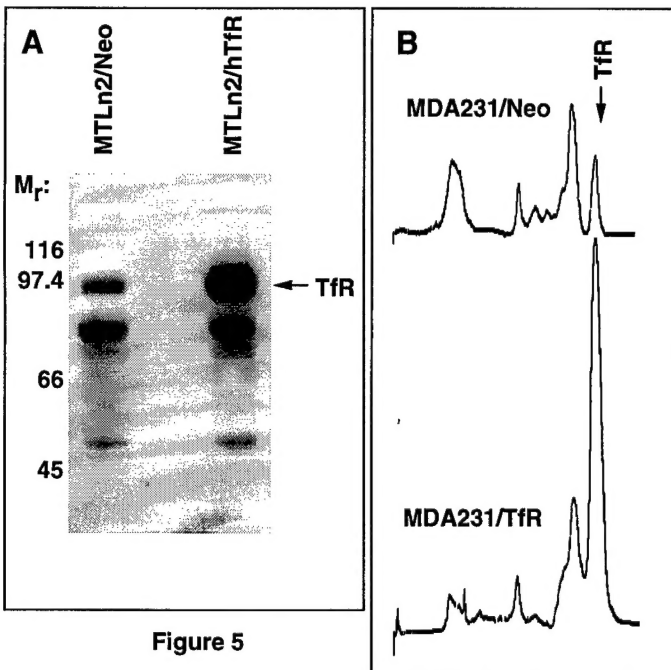


Figure 5

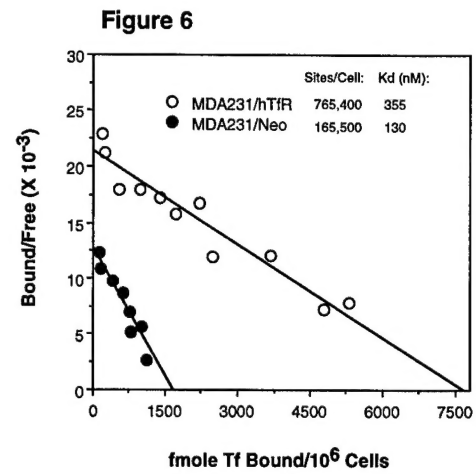


Figure 7

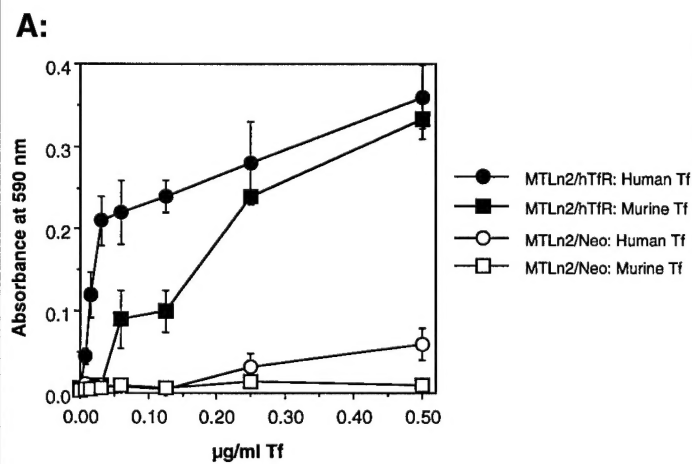
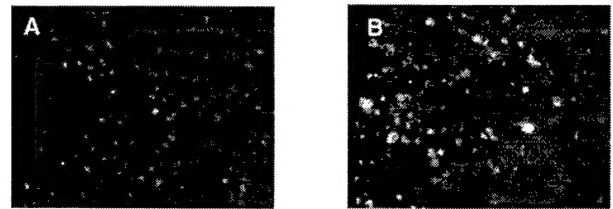


Figure 8

Figure 9

